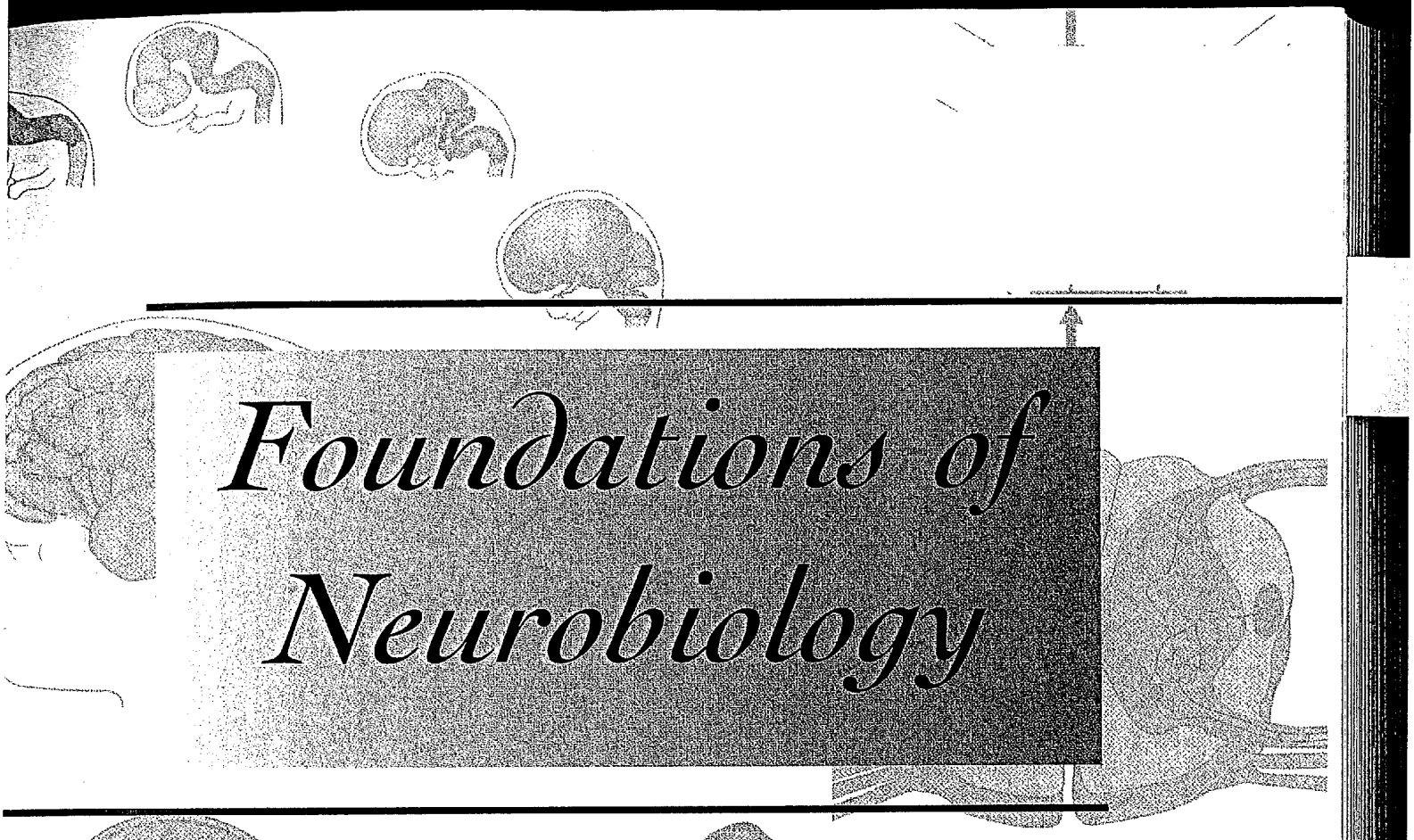

Foundations of Neurobiology

Fred Delcomyn

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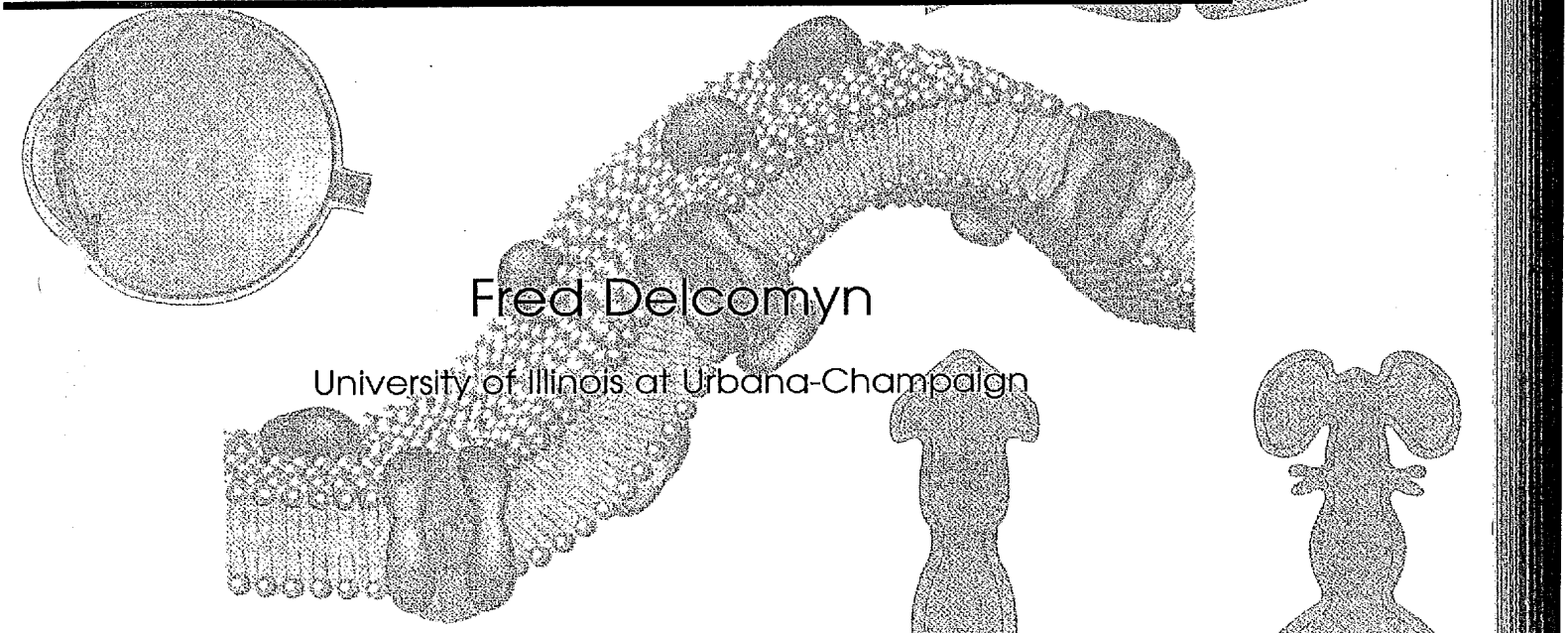

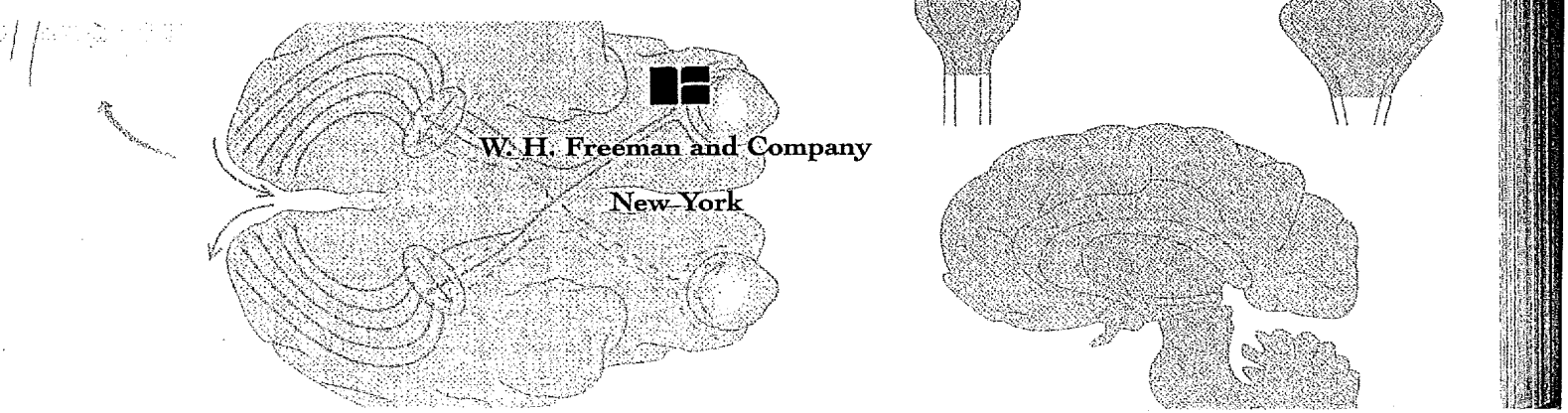

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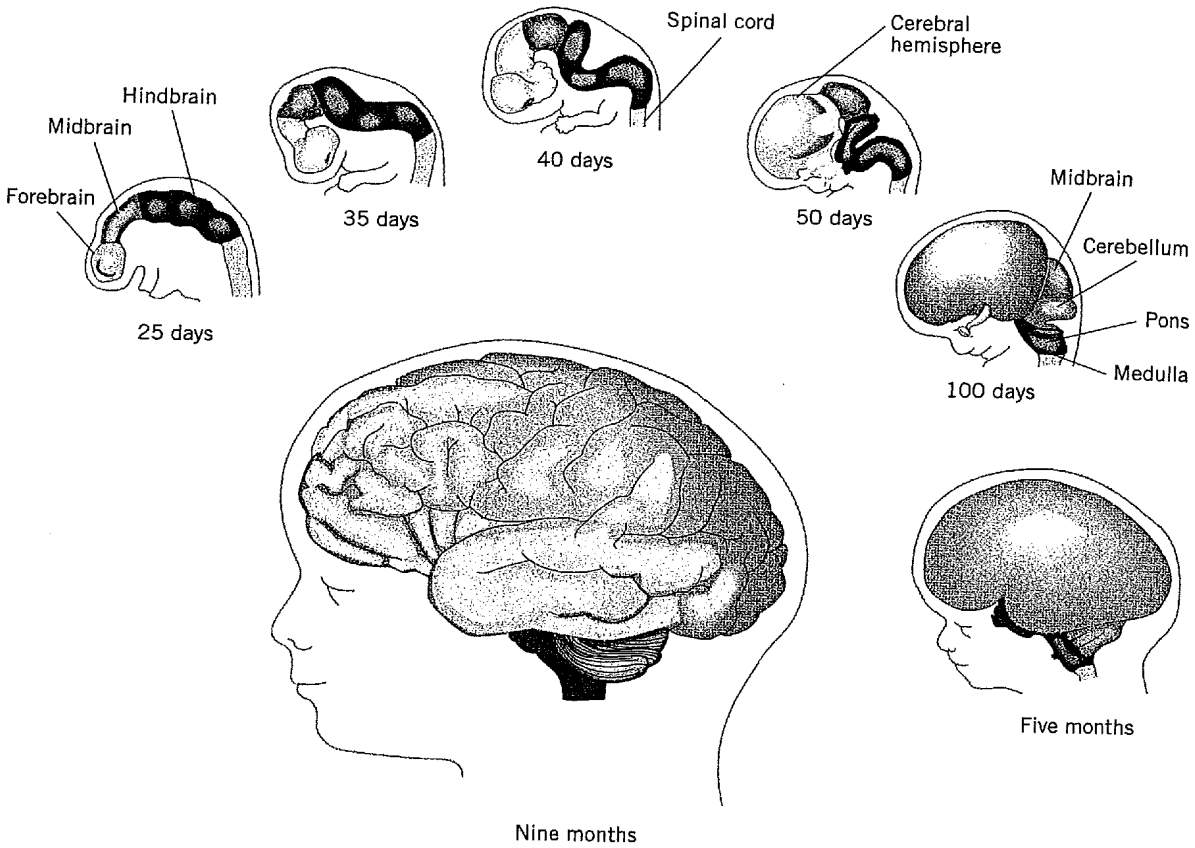


FIGURE 3-12. The development of the human brain. As the neural tube differentiates, it folds and bends on itself. The main lobes of the brain develop last.

the interior of the brain, the diencephalon and its two main constituent structures, the hypothalamus and the thalamus, remain intact, perched atop the midbrain.

Rapid growth of specific regions of the embryonic brain causes formation of the cerebellum on top of the hindbrain and of the cerebral hemispheres, which cover virtually all the brain structures beneath it.

The Lower Brain

When you look at the nervous system in a vertebrate embryo and at the brain of a relatively simple vertebrate like a fish, it is easy to see the fundamentally linear arrangement of the spinal cord and the brain. Even in the highly developed mammalian brain, the medulla, the pons, and the midbrain are arranged in a row, one after the other (Figure 3-13). By analogy with the straight stem of a plant with a flower perched on top, these parts of the brain can be thought of as the stem that supports the flower represented by the cerebral hemispheres. The analogy is particularly apt because these linearly arranged parts of the brain are collectively referred to as the **brain stem**. The cerebellum, because it sits perched above the pons rather than being in line with the other parts of the hindbrain, is not included in the brain stem. Based on its embryological origin, however, it is part of the hindbrain. The parts of the brain stem all contribute to the regulation of basic bodily functions such as respiration and heartbeat.

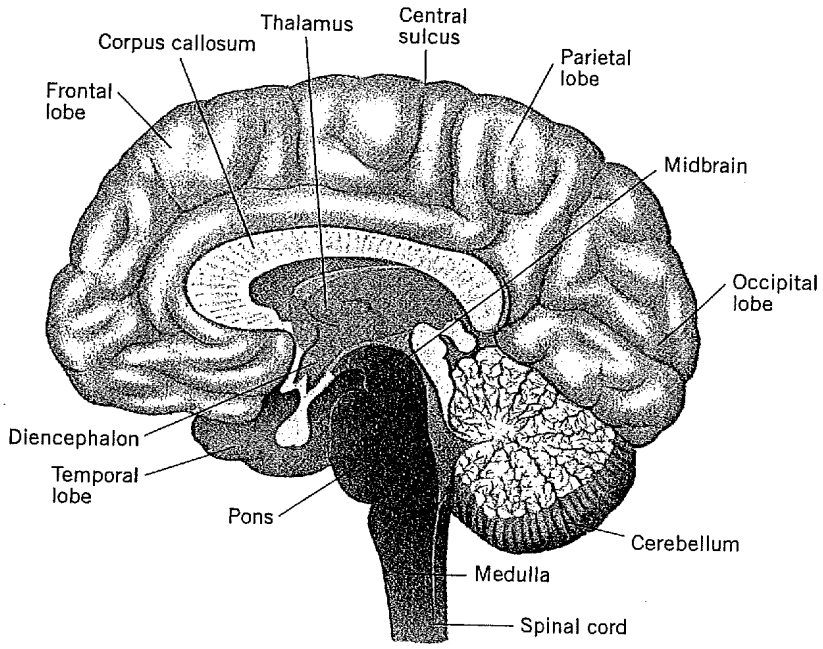


FIGURE 3-13. Section through the middle of the human brain, showing the medulla, pons, and midbrain (collectively the brain stem) in relation to some of the other main structures of the human brain. Some of the labeled parts are discussed in later sections of this chapter.

The Medulla and the Pons

Two components of the hindbrain are part of the brain stem. The medulla arises directly from the spinal cord, and looks somewhat like an enlargement of the cord. Joined to the anterior end of the medulla is the pons, a bulbous part of the brain stem that is distinctive for the fluting of its external surface (see Figure 3-9). From an evolutionary point of view, these parts of the hindbrain constitute the oldest part of the brain. In line with its primitive origins, many parts of the hindbrain control what you might think of as the "primitive" functions in the animal's body, those that are fundamental to life and not under conscious control. Both the medulla and the pons contain important nuclei, control centers containing cell bodies and dendrites of neurons that help to control vital functions like breathing, heart rate, and blood pressure, and that are responsible for initiating the reflexive actions of coughing, gagging, and vomiting.

Some of the medullary and pontine nuclei are associated with **cranial nerves**. These nuclei contain the cell bodies of motor neurons that leave the brain via some of the cranial nerves. Like spinal nerves, cranial nerves serve as the communication links between the CNS, the brain in this case, and the rest of the body. However, whereas spinal nerves are all organized in the same pattern (dorsal roots being sensory, ventral roots being largely motor), not all cranial nerves are the same. Some are efferent, carrying motor information to muscles and glands; some are afferent, bringing sensory information into the brain; and some are mixed, carrying both efferent and afferent fibers. Not all cranial nerves connect with the medulla and pons; some also connect to the mesencephalon and the forebrain. The locations of the 12 pairs of cranial nerves are shown in Figure 3-14, and their functions and the type of information they carry are listed in Table 3-3.

In addition to housing important control nuclei, the brain stem is also a major pathway for communication within the CNS. Some of the nerve tracts found there connect one nucleus in the brain stem to another, some connect nuclei in the brain stem to the

TABLE 3-3. The Cranial Nerves

Nerve	Function	Origin of Motor Nerves	Destination of Sensory Nerves
I Olfactory	Sensory: olfactory input		Olfactory bulb
II Optic	Sensory: visual input		Lateral geniculate nucleus (thalamus)
III Oculomotor	Motor: controls most eye muscles	Mesencephalon	
IV Trochlear	Motor: controls superior oblique eye muscles	Mesencephalon	
V Trigeminal	Mixed: carries sensory input from the face; controls muscles that move the jaw	Pons	Mesencephalon, pons, and medulla
VI Abducens	Motor: controls external rectus eye muscles	Pons	
VII Facial	Mixed: carries sensory input from tongue and palate; controls muscles of the face	Pons	Pons and medulla
VIII Vestibulocochlear (vestibular, cochlear)	Sensory: carries auditory input and input concerning balance		Pons and medulla
IX Glossopharyngeal	Mixed: carries sensory input from the tongue and throat; controls throat muscles	Medulla	Pons and medulla
X Vagus	Mixed (autonomic): carries sensory input from the heart, lungs, and viscera; controls movements of the heart, lungs, and viscera	Medulla	Pons and medulla
XI Accessory	Motor: controls neck muscles	Medulla	
XII Hypoglossal	Motor: controls tongue and neck muscles	Medulla	

spinal cord and to nuclei in the rest of the brain, and some go right through the brain stem, connecting spinal centers to nuclei in the midbrain and forebrain. Tracts that interconnect nuclei within the brain stem are important for the proper execution of many vital reflexes.

Among the most prominent of the tracts that course through the brain stem are the two ventrally located **pyramidal tracts**. These tracts, which derive their name from the pyramid-shaped part of the medulla through which they travel (Figure 3-15), carry axons that convey motor information from the motor centers in the forebrain to the motor centers in the spinal cord. Tracts of this type, which carry information from the cortex to the spinal cord, are called **corticospinal tracts**. Tracts conveying information in the opposite direction are called **spinocortical tracts**.

Distributed throughout the brain stem is a loose network of neurons called the **reticular formation**, or **reticular activating system**. Neurons of this network form a number of biochemically and morphologically distinct groups within the brain stem, groups that communicate extensively with one another as well as with other parts of the brain. Researchers have identified four important

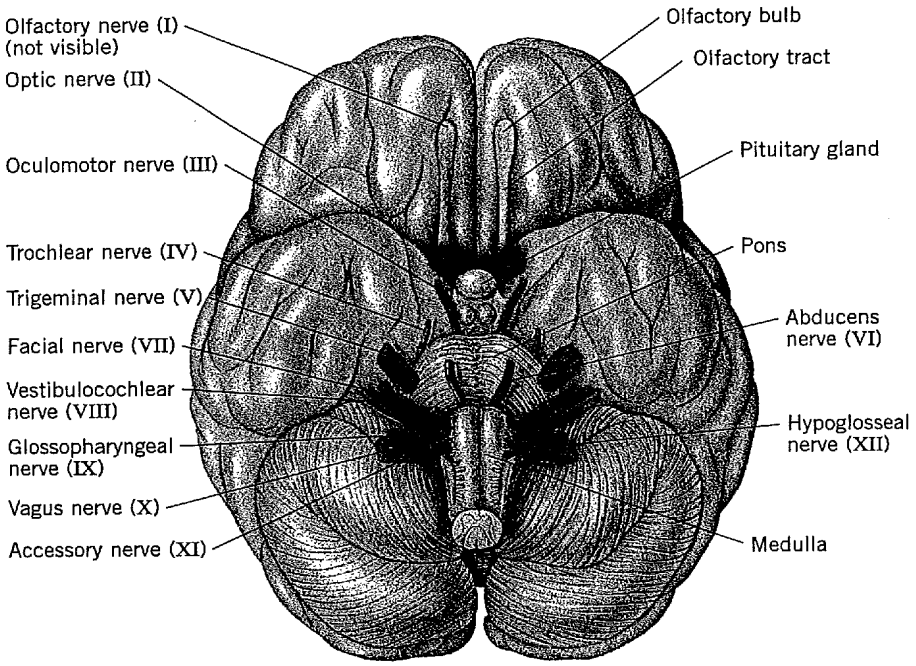


FIGURE 3-14. The cranial nerves of the human brain, shown in ventral view. Several nerves have origins or destinations in more than one part of the brain.

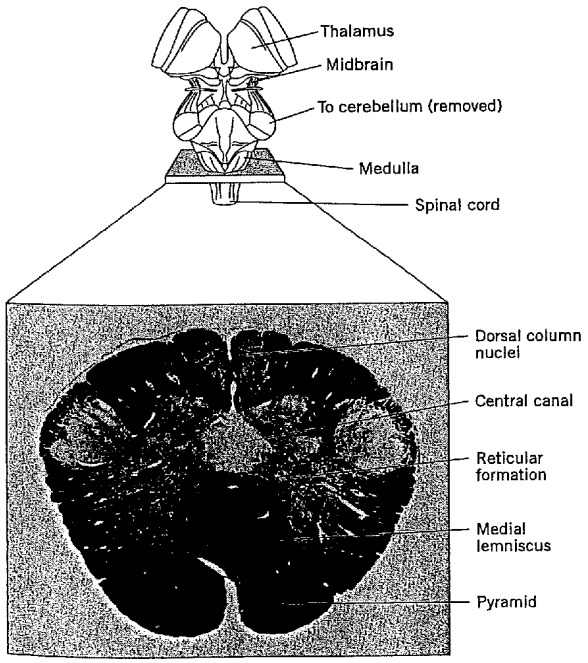


FIGURE 3-15. Photomicrograph of a section through the human medulla. The drawing, a dorsal view of the brain stem, shows the level of the section in a dorsal view.

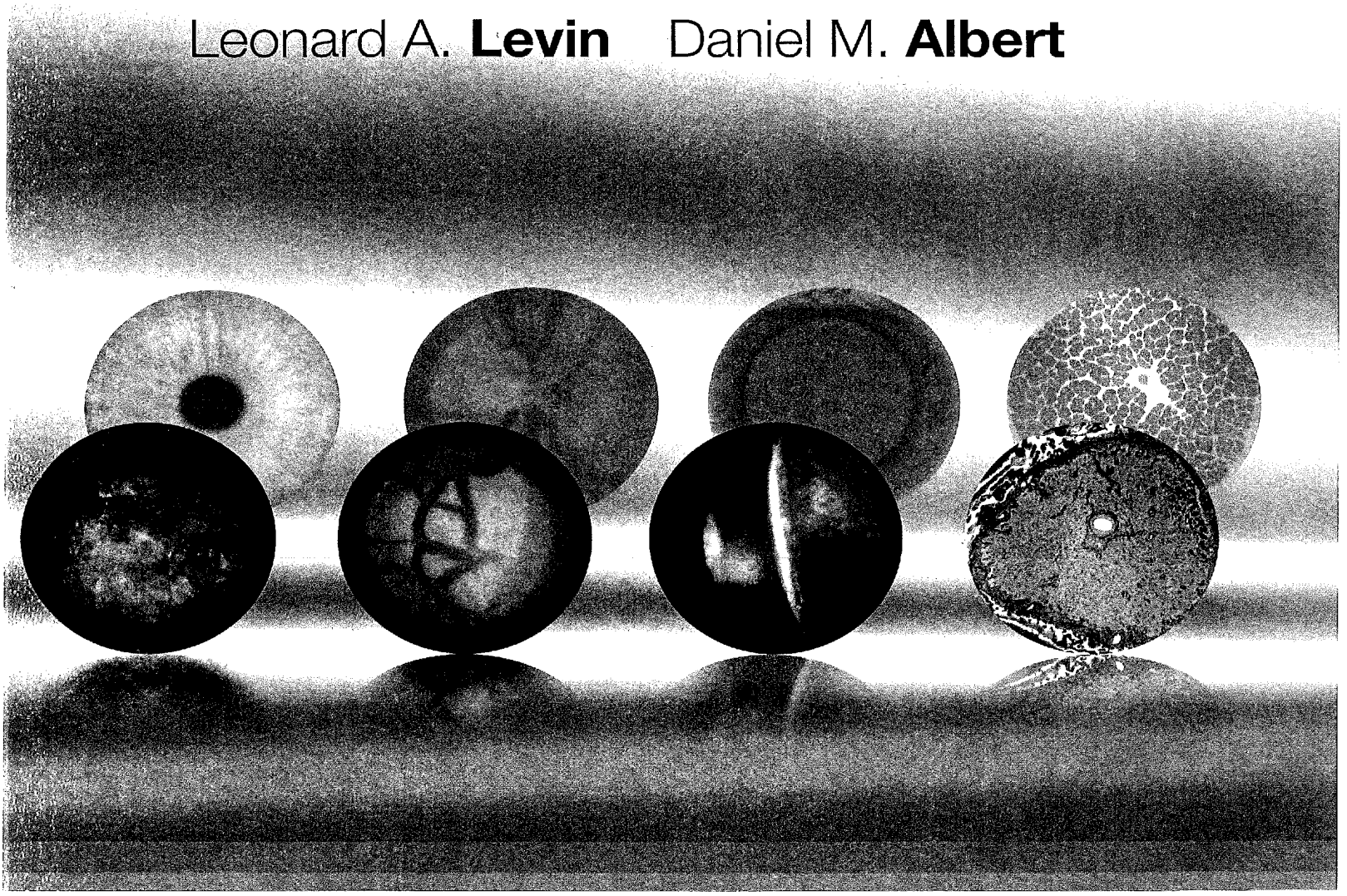
functions of the system. These are (1) to modulate the sensation of pain; (2) to modulate certain postural reflexes and muscle tone; (3) to help control breathing and heartbeat; and (4) to regulate the level of brain arousal and, in humans, consciousness. The last function is that with which the reticular formation is the most closely associated. For example, the **raphe nuclei** that lie along the midline of the medulla, pons, and midbrain are especially important in maintaining wakefulness (Figure 3-16). Damage to them can result in permanent coma.

All sensory input that enters the brain via the medulla is also sent to neurons of the reticular formation. These may monitor sensory input for importance, and alert higher brain centers when critical input is detected. It has been suggested that the reticular formation is able to do this in part because it receives input from the cortex and uses that input as the basis for its decisions.

Ocular Disease

MECHANISMS AND MANAGEMENT

Leonard A. **Levin** Daniel M. **Albert**



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Ocular Disease: Mechanisms and Management

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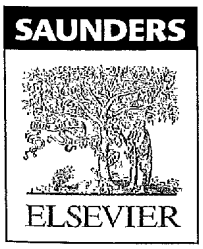

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CHAPTER 3

Wound healing after laser in situ keratomileusis and photorefractive keratectomy

Fabricio Witzel de Medeiros and Steven E Wilson

Clinical background

The safety and predictability of laser in situ keratomileusis (LASIK) and photorefractive keratectomy (PRK) have improved since these procedures were introduced, but the corneal wound-healing response remains a major contributor to variability of results following these procedures. Corneal wound healing entails the complex interactions of different cellular types, including corneal epithelial cells, keratocytes, and, possibly, endothelial cells, in addition to corneal fibroblasts, myofibroblasts, inflammatory cells, lacrimal gland cells, and others. In large part, this communication is mediated by soluble growth factors, cytokines, and chemokines via membrane-bound and soluble receptors.[1,2]

The unwounded adult cornea is a transparent and avascular structure, providing not only the major refractive surface involved in visual image transmission, but also a protective barrier against external injuries, including microbial infections that are potentially vision-threatening. Activation of these systems during refractive surgery can result in the deposition of opaque fibrotic repair tissue and, possibly, scarring. In order to understand and control these complex interactions better and improve the results and safety of LASIK and PRK, it is important to have a basic understanding of normal and abnormal corneal wound-healing responses. This chapter provides a framework that will allow the clinician not only to understand these interactions, but also at least partially to control them through surgical technique and rational application of medications.

Pathophysiology and pathology

The normal wound-healing response

Corneal stromal fibrils and other matrix components are precisely organized to provide transparency essential to corneal function. However, cellular repair processes during corneal healing can disturb this architecture and lead to visual impairment. The corneal wound-healing response involves a complicated balance of cellular changes, including cell death (apoptosis and necrosis), cell proliferation, cell motility, cell differentiation, expression of cytokines, growth factors, chemokines and their receptors, influx of inflammatory cells, and production of matrix materials (Box 3.1). In large part, communications between corneal cells, nerves, inflammatory cells, bone marrow-derived cells, and other cells are the critical determinants of normal and abnormal corneal wound-healing responses. Although many of these interactions occur simultaneously, for discussion purposes it is convenient to describe the wound-healing response as a pathway, similar to glycolysis or the Kreb's cycle.

Corneal epithelial injury is a common initiator of the corneal wound-healing response to refractive surgical procedures, as well as in trauma and some diseases. Here we will concern ourselves only with surgical injury associated with LASIK and PRK. Corneal epithelial injury triggers the release of a variety of cytokines, such as interleukin-1 (IL-1)-α and -β, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, platelet-derived growth factor (PDGF), and epithelial growth factor (EGF), that regulate keratocyte apoptosis, proliferation, motility, differentiation, and other functions during the minutes to months after surgical insult.[1,2] In turn, once stimulated by these epithelial-derived soluble factors via membrane-bound receptors, keratocytes not only alter cellular functions, but also produce other soluble modulators that regulate corneal epithelial proliferation and migration (hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF)), attract inflammatory cells (granulocyte chemotactic and stimulating factor (G-CSF), monocyte chemotactic and activating factor (MCAF), neutrophil-activating peptide (ENA-78)), and other corneal changes.[3–7] Collagenases, metalloproteinases, and other enzymes are activated and released in the stroma during the wound-healing response and function to degrade, remove, and regenerate damaged tissue.[8] The expression of these collagenases and metalloproteinases by keratocytes and corneal fibroblasts is also regulated by IL-1 and fibroblast growth factor-2 derived from the injured corneal epithelial cells.[9]

A recurring theme that must be appreciated to understand corneal wound healing is ongoing communication between epithelial cells and stromal cells mediated by soluble cytokines and chemokines. These interactions occur imme-

diately after injury and continue for weeks, months, or occasionally even years, for example with persistence of haze following PRK.

Many growth factors released during the corneal wound-healing response can be derived from more than one cell type and regulate more than one process. EGF can be used to illustrate this principle. EGF is produced by epithelial cells, keratocytes, corneal fibroblasts, lacrimal cells, and, possibly, other cells. EGF regulates corneal epithelial cell proliferation, motility, and differentiation.[1,2] EGF also triggers the formation of new hemidemosomes on epithelial cells after injury.[6,7,10] EGF also has influence on the proliferation of limbal cells that migrate toward the injury site to seal the wound and to reform a normal stratified epithelial layer.[11,12] In addition, different growth factors may regulate a single function. For example, EGF, HGF, and KGF all regulate corneal epithelial proliferation.[1,2] The effect that predominates at a particular point in the wound-healing response likely depends on factors such as receptor expression, cellular localization, cellular differentiation, and the influences of interacting networks of soluble and intracellular factors.

Box 3.1 Key processes in the corneal wound-healing response

- Epithelial injury
- Stromal cell death (apoptosis and necrosis)
- Influx of inflammatory cells
- Cell proliferation
- Cell motility
- Cell differentiation
- Release of cytokines, growth factors, chemokines, and expression of their receptors
- Production of extracellular matrix materials
- Epithelium healing

Epithelial injury is typically the initiator of the wound-healing response associated with corneal surgery or injury. For example, epithelial scrape or epithelial ethanol exposure associated with PRK or laser epithelial keratomileusis (LASEK), respectively, epithelial blade penetration associated with Epi-LASEK or LASIK are initiators of corneal wound healing that result in the release of IL-1α, IL-1β, TNF-α, and a host of other modulators that alter the functions of keratocytes, inflammatory cells, and the epithelial cells themselves. Similarly, damage to the epithelium at the edge of the flap in femtosecond LASIK flap formation triggers the wound-healing cascades, although the femtosecond laser has direct stromal necrotic effects that influence the overall wound-healing response of surgery performed with this procedure,[13] as will be covered later.

Apoptosis and necrosis in initiation, modulation, and termination of wound healing (Box 3.2)

The first stromal change that is noted following epithelial injury is apoptosis of the underlying keratocyte cells (Figure 3.1). Apoptosis, or programmed cell death, is a gentle, regulated form of cell death that occurs with the release of only limited intracellular components such as lysosomal enzymes that would potentially damage surrounding tissue.[14] Keratocytes undergoing apoptosis are found to have chromatin

Box 3.2 Apoptosis and necrosis in initiation, modulation, and termination of wound healing

- Apoptosis of the underlying keratocyte cells
- Modulation by eliminating excess inflammatory, fibroblast, and other cells
- Elimination of myofibroblasts

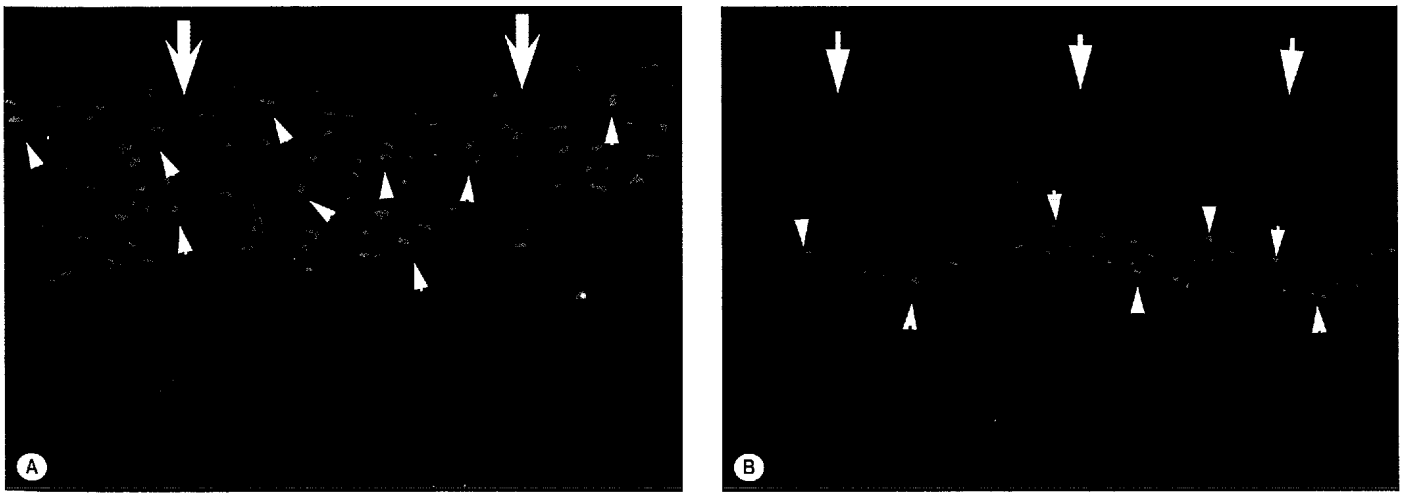

Figure 3.1 Keratocyte apoptosis detected with the terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay at 4 hours after photorefractive keratectomy (PRK) or laser in situ keratomileusis (LASIK). Note that after PRK (A, 600× magnification) keratocytes undergoing apoptosis (arrowheads) are located in the anterior stroma. Arrows in (A) indicate the anterior stromal surface. After LASIK (B, 200× magnification) keratocytes undergoing apoptosis (arrowheads) are localized in the deeper stroma anterior and posterior to the lamellar cut. The epithelium in (B) is indicated by arrows.

condensation, DNA fragmentation, cell shrinkage, and formation of membrane-bound vesicles called apoptotic bodies that contain intracellular contents. The localization of the apoptosis response is related to the type of injury, and in large part determines the localization of the subsequent wound-healing events. For example, in PRK, LASEK, and Epi-LASEK, keratocyte apoptosis occurs in the anterior stroma beneath the site of epithelial injury (Figure 3.1A). In contrast, keratocyte apoptosis associated with microkeratome LASIK occurs at the site of blade penetration at the edge of the flap and along the lamellar cut in the central stroma (Figure 3.1B).

The apoptosis process is likely regulated by soluble cytokines such as IL-1 and TNF-α released from injured epithelial cells and the Fas-Fas ligand system expressed in keratocytes.[1,2] Apoptosis is an extremely rare event in unwounded normal cornea. Once an injury to the epithelium occurs, however, keratocytes undergoing apoptosis can be detected within moments.[14,15] This early wave of relatively pure apoptosis makes a transition into a later phase in which both apoptosis and necrosis occur in many stromal cells, including keratocytes, corneal fibroblasts, and invading inflammatory cells. Although all of these cells are typically labeled with the terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, careful analysis with transmission electron microscopy demonstrates that cellular necrosis, a more random death associated with release of intracellular enzymes and other components, also makes a major contribution.[15] It is unknown whether necrosis that occurs during corneal wound healing is a regulated event or merely a result of cells being killed by inflammation or other contributors to healing. A much later low-level phase of apoptosis occurring in myofibroblasts is also noted in corneas that develop haze.

Precise regulation of the apoptosis processes that occur during corneal wound healing implies an important function besides a merely reactionary response to the injury.[16] Studies have suggested that the earliest apoptosis response is likely a defense mechanism designed to limit the extension of viral pathogens, such as herpes simplex and adenovirus, into the stroma and eye after initial infection of the corneal epithelium.[17] The second phase of stromal apoptosis extending from hours to a week after injury likely functions to modulate the corneal wound-healing response by eliminating excess inflammatory, fibroblast, and other cells. The latest phase of stromal apoptosis that occurs in corneas with haze serves to rid the stroma of myofibroblasts that are no longer needed.[17]

Mitosis and migration of stromal cells

Mitosis and migration of stromal cells are noted approximately 8–12 hours after the initial corneal injury.[13] Initially, most cells undergoing mitosis appear to be keratocytes, but corneal fibroblasts and other cells may make subsequent contributions to this response. This cellular mitosis response provides corneal fibroblasts and other cells that participate in corneal wound healing and replenish the stroma. Once again, localization of the stromal mitosis response is related to the type of injury. Thus, in PRK stromal mitosis tends to occur in the anterior stroma, as well as in the peripheral and posterior stroma outside the zone of apoptosis (Figure 3.2).

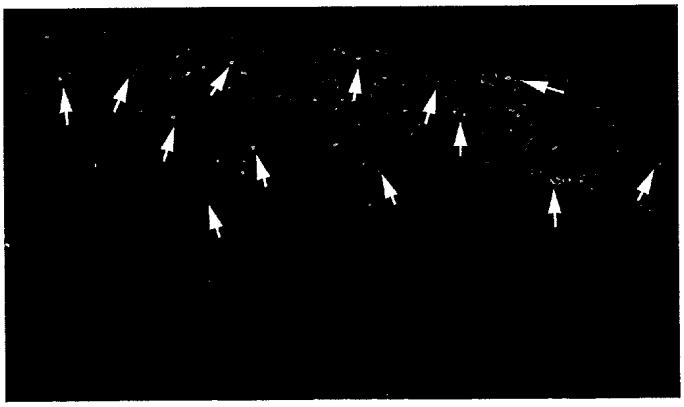

Figure 3.2 Stromal cell mitosis at 24 hours after photorefractive keratectomy. Arrows indicate cells in the stroma that stain for Ki-67, a marker for mitosis. Blue is the 4',6-diamidino-2-phenylindole (DAPI) stain for the nucleus that stains all cells. 500× magnification.

In LASIK, stromal mitosis occurs at the periphery of the flap where the epithelium was injured, and anterior and posterior to the lamellar cut.

Mitosis and migration of stromal cells are regulated by cytokines released from the epithelium and its basement membrane. For example, PDGF is produced by corneal epithelium and bound to basement membrane due to heparin-binding properties of the cytokine. It is released from the epithelial basement membrane after injury and stimulates mitosis of corneal fibroblasts. It is also highly chemotactic to corneal fibroblasts, tending to attract them to the source of the cytokine. Thus, in PRK, for example, PDGF released from the injured epithelium and basement membrane stimulates surviving keratocytes in the peripheral and posterior stroma to undergo mitosis and the daughter cells are attracted to the ongoing PDGF release and repopulate the anterior stroma. Other cytokines such as TGF-β also likely contribute to this keratocyte/corneal fibroblast mitosis and migration.[2]

Corneal fibroblasts derived from keratocytes produce collagen, glycosaminoglycans, collagenases, gelatinases, and metalloproteinases[18] used to restore corneal stromal integrity and function. These cells also produce cytokines such as EGF, HGF, and KGF that direct mitosis, migration, and differentiation of the overlying healing epithelium.[1,2,19] After total epithelialization, the fibronectin clot disappears and the nonkeratinized stratified epithelium is re-established.[11,12,20–22]

Inflammatory cell influx (Box 3.3)

Beginning approximately 8–12 hours after the initial epithelial injury, and lasting for several days, a wave of inflammatory cells migrates into the cornea (Figure 3.3) from the limbal blood vessels and tear film.[23,24] These cells function to clear cellular and other debris from the injury and to respond to pathogens that could be associated with injuries such as viral or bacterial infections.

The inflammatory cells that sweep into the cornea are chemotactically attracted into the stroma by cytokines and chemokines released directly by the injured epithelium and induced in keratocytes and corneal fibroblasts by cytokines

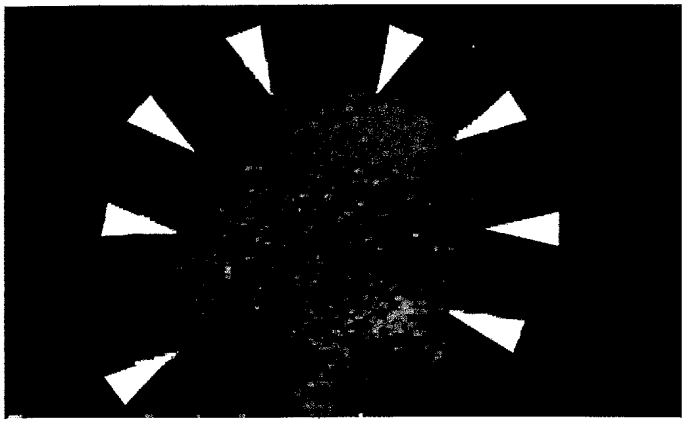

Figure 3.3 At 24 hours after epithelial scrape, as performed in photorefractive keratectomy, thousands of bone marrow-derived cells invade the cornea in a chimeric mouse with fluorescent green protein-labeled, bone marrow-derived cells. Magnification 10x.

Box 3.3 Inflammatory cell influx

- Inflammatory cell migration
- Clear cellular and other debris
- Varies with type of injury

released from the epithelium. IL-1 appears to be the master regulator of this response since corneal fibroblasts produce dozens of proinflammatory chemokines in response to IL-1 binding to IL-1 receptors on the stromal cells.[23]

The pattern of entry of the inflammatory cells into the central cornea may differ depending on the type of injury. In PRK and other surface ablation procedures the cells tend to be fairly equally distributed across the anterior to mid stroma. In LASIK, however, many of the cells enter along the lamellar cut since this is the path of least resistance. In the LASIK procedure, augmented release of epithelial IL-1, for example, with epithelial slough caused by a microkeratome, triggers massive influx of cells along the lamellar cut and produces the disorder diffuse lamellar keratitis.[25] Since the potential space produced by the lamellar cut persists for years following LASIK, epithelial trauma even many years later may precipitate diffuse lamellar keratitis.

Completion of the healing response (Box 3.4)

As the corneal wound-healing response is completed, excess cells are eliminated by apoptosis and necrosis, and the keratocyte cells that were lost are replenished by mitosis and migration of keratocytes that did not undergo apoptosis. In the normal cornea that does not develop haze, most of these stromal processes appear to be completed within 1–2 weeks after injury, as long as the integrity of the epithelium is re-established. In eyes with persistent epithelial defects, cytokine triggers from the epithelium continue, along with stromal apoptosis, necrosis, and mitosis, eventually leading to destruction of the stroma and perforation if the epithelium does not heal.

In corneas where the epithelium heals normally, there may be persistent epithelial hyperplasia and/or hypertrophy

Box 3.4 Completion of healing response

- Elimination of excess cells by apoptosis and necrosis
- Replenishment by mitosis and migration of keratocytes
- Healing time in 1–2 weeks of epithelium re-established
- Perform enhancement procedures after refractive stability

Box 3.5 Altered healing in corneas that develop haze

- Development of haze in the cornea correlates with the appearance of myofibroblast cells
- Sustained exposure of transforming growth factor-β, and possibly other cytokines required for development and persistence of myofibroblasts
- Defective regeneration of the basement membrane commonly associated with surface irregularity, possibly genetic influences, and other factors

that may mask the full refractive correction.[15] Thus, a cornea that appears to be undercorrected after PRK or LASIK for myopia may have a portion of the attempted correction masked by a temporary thickening of the epithelium. At the molecular level, this could result from excess penetration and binding of EGF, HGF, KGF, and other cytokines to the epithelial receptors. The higher levels of epithelium-modulating cytokines are likely derived from fibroblasts "activated" during the wound-healing response in the stroma. Once the wound-healing response subsides and the stromal cells return to their normal metabolic activity, the levels of these cytokines diminish and the epithelial architecture is restored. This points out the importance of waiting to perform enhancement procedures until there is refractive stability. The length of time required likely varies with the individual patient.

Etiology and treatment of wound healing-associated corneal abnormalities

Altered healing in corneas that develop haze (Box 3.5)

After surface ablation, including PRK, LASEK, and Epi-LASEK, depending on the level of attempted correction, a proportion of corneas develop trace to severe stromal opacity, termed haze.[26,27] The higher the attempted correction, the greater the percentage of corneas that develop haze and the greater the incidence of severe haze associated with regression of the refractive correction and decreased vision (Figure 3.4A). Rarely, central haze can also occur in LASIK, typically associated with severe diffuse lamellar keratitis, buttonhole, or other abnormal flaps. Marginal haze at the flap margin, where the microkeratome or femtosecond laser penetrated the epithelium, is common.

The development of haze in the cornea correlates with the appearance of myofibroblast cells in the anterior stroma (Figure 3.4B) beneath the epithelial basement membrane.[15]

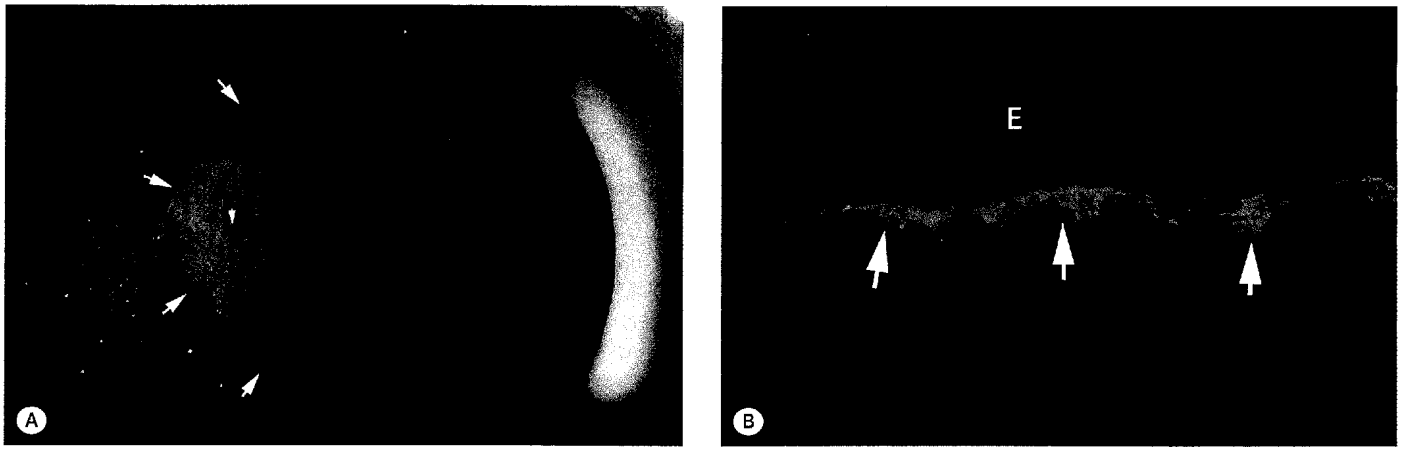

Figure 3.4 Haze and myofibroblasts. (A) Slit-lamp photograph of severe corneal haze in an eye that had photorefractive keratectomy (PRK) for –9 D of myopia at 12 months after surgery. Arrows indicate the border of haze at the edge of the ablation. Small arrowhead indicates an area of early clearing of haze, termed a lacuna. (B) In a rabbit eye that had PRK for –9 D of myopia there are large numbers of myofibroblasts (arrows) that stain green for α-smooth-muscle actin. The myofibroblasts are located immediately beneath the epithelium (E). Magnification 600×.

Myofibroblasts are themselves opaque, due to diminished production of corneal crystallins.[28–30] In addition, these cells are active factories that produce collagen and other matrix materials that do not have the normal organization associated with corneal stromal transparency.

The earliest appearance of myofibroblasts after PRK, detected with the α-smooth muscle actin marker, is noted approximately 1 week after surgery.[15,31] Sustained exposure to TGF-β, and possibly other cytokines, derived primarily from the epithelium, is required for development and persistence of myofibroblasts.[15,31–33] If the basement membrane of the healing epithelium is regenerated with normal structure and function, penetration of TGF-β into the stroma is limited and only small numbers of myofibroblasts are generated and persist.[31] Defective regeneration of the basement membrane, however, commonly associated with surface irregularity, possibly genetic influences, and other factors, leads to ongoing penetration of TGF-β and development of large numbers of persistent myofibroblasts and haze, typically immediately below the epithelium.[31]

The identity of the progenitor cell(s) for the myofibroblast in the corneal stroma remains uncertain. Myofibroblasts can be generated from corneal fibroblasts in vitro under proper culture conditions, including availability of TGF-β.[18,32,33] However, in other tissues, myofibroblasts have also been shown to develop from bone marrow-derived cells.[34,35] A dual origin for myofibroblasts could provide an explanation for haze being corticosteroid-responsive in some corneas and corticosteroid-unresponsive in others.

Haze typically persists for 1–2 years after surgery and then slowly disappears over a period of months or years. This time course, however, may be significantly prolonged in corneas treated with mitomycin C, which subsequently develop "breakthrough haze." When haze finally disappears, it is likely that the slow repair of the epithelial basement membrane, and restoration of basement membrane barrier function, eventually results in diminished penetration of TGF-β into the stroma to a level insufficient to maintain myofibroblast viability, and the cells undergo apoptosis.[31] This is followed by reabsorption and/or reorganization of myofibroblast-produced collagens and other matrix materials by keratocytes. Thus, there is a slow restoration of stromal transparency.

Mitomycin C treatment to prevent haze

Mitomycin C is a chemotherapeutic agent with cytostatic effects that is applied topically to the stromal surface to prevent haze after PRK. Mitomycin C blocks RNA/DNA production and protein synthesis. This results in inhibition of the cell proliferation, and presumably reduces the formation of progenitor cells to myofibroblasts.[36] The resulting effect in diminishing haze has been confirmed in clinical studies.[37] Although mitomycin C at the lower concentrations of 0.002% decreases haze formation in animal studies,[36] there tends to be a higher incidence of "breakthrough haze" and, therefore, the higher concentration of 0.02% for 30–60 seconds has once again become the most commonly used.

Some surgeons restrict mitomycin C use to corrections greater than 5–6 D of myopia. Although rare, haze is seen in lower corrections that are not treated with mitomycin C. In addition, most refractive surgeons use mitomycin C for any eye that has PRK after previous surgery, including PRK, LASIK, radial keratotomy, and corneal transplantation.

Corneas treated with mitomycin C have a lower anterior stromal keratocyte density than corneas that are not treated with mitomycin C.[36] This effect persists for at least 6 months after treatment in animal models. It is not known whether there will be long-term effects from diminished keratocyte maintenance of the stroma decades after surgery.

Altered wound healing in femtosecond LASIK

Recent studies have demonstrated that the femtosecond laser directly triggers necrosis of keratocytes anterior and posterior to the lamellar cut.[13] This results in greater inflammatory cell infiltration into the stroma during the early wound-healing response and, therefore, greater inflammation. Stromal necrosis is proportional to the amount of femtosecond laser energy used to generate the cut, especially with earlier models

Box 3.6 Nerves and corneal wound-healing response

- Damage to corneal nerves diminishes epithelial viability
- Neurotrophic factors needed for epithelial homeostasis
- Laser-induced neurotrophic epitheliopathy continues until nerves regenerate into the flap
- Photorefractive keratectomy damage to nerve terminals resolves more quickly than laser-assisted intrastromal keratoplasty (LASIK)
- Ciclosporin A may be of benefit

of the femtosecond laser, such as the 15 kHz Intralase (Irvine, CA). This effect is diminished with more recent models, including the 30 kHz and 60 kHz Intralase models. However, even with these more efficient lasers, it is prudent to use the minimum energy level that yields a flap that is easy to lift. In our experience, 1.0 μJ settings with the 60 kHz Intralase for both the lamellar and side cuts yield similar inflammation to LASIK performed with a microkeratome.

Nerves and the corneal wound-healing response (Box 3.6)

Disorders that damage the corneal nerves may diminish corneal epithelial viability and lead to neurotrophic ulceration. Corneal nerves have important influences on corneal epithelial homeostasis through the effects of neurotrophic factors like nerve growth factor and substance P. These neurotrophic factors have been shown to accelerate epithelial healing in vivo.[38] After LASIK corneas often develop a neurotrophic epitheliopathy characterized by punctate epithelial erosions on the flap with only marginal decreases in tear production.[39] This condition has been termed LASIK-induced neurotrophic epitheliopathy (LINE).[39] The condition typically presents from 1 day to 1 month following LASIK and continues for 6–8 months, until the nerves regenerate into the flap. Many patients who develop severe LINE probably have an underlying tendency towards chronic dry eye and often benefit from treatment with topical ciclosporin. In our experience, LINE is less common and less severe after femtosecond LASIK with 100-μm thick flaps, presumably because thinner flaps result in less corneal nerve damage (Medeiros and Wilson, unpublished data, 2007).

PRK also damages the corneal nerve terminals. Neurotrophic epithelial after PRK may occasionally be problematic, but tends to resolve more quickly than after LASIK. Topical ciclosporin A may also be of benefit in these patients.

Conclusions

The corneal wound-healing response, and the complex cellular interactions associated with it, are major determinates of the response of corneas to surgical procedures, including LASIK and PRK. An understanding of these interactions is important to optimize surgical outcomes and limit complications.

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Declaration of interest
Dr. Medeiros has no proprietary or financial interest in any materials or methods described in this chapter. Dr. Wilson is a consultant to Allergan, Irvine, CA.

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A complete list of chapter references is available online at www.expertconsult.com. See inside cover for registration details.

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CHAPTER 42

Optic nerve axonal injury

Daniela Toffoli and Leonard A Levin

Overview

Unlike the peripheral nervous system (PNS), in which axonal injury leads to regeneration, injury to axons of central nervous system (CNS) neurons is irreversible, and usually leads to death of the cell body. The optic neuropathies, which are the main focus of this chapter, almost always involve injury to the retinal ganglion cell (RGC) axon. Etiologies include glaucoma, ischemia, compression, inflammation and demyelination, transection, infiltration, and papilledema (Box 42.1). Each of these involves axonal injury that ultimately leads to varying degrees of ganglion cell death.

Glaucoma (Chapters 19–29) is the most common of all of the optic neuropathies, ischemic optic neuropathy (Chapters 41 and 43) is the most common acute optic neuropathy in older persons, and optic neuritis (Chapter 37) is most common in the young. Compressive etiologies include neoplasms, aneurysms, and enlargement of extraocular muscles in thyroid-associated orbitopathy. Transection of the optic nerve may be partial or complete, and can occur with trauma such as bullet or knife wounds, or iatrogenically, e.g., during resection of a tumor. Infiltration of the optic nerve may involve neoplasm (e.g., gliomas or metastases) or inflammation (e.g., sarcoidosis) and usually results in a combination of compressive, inflammatory, and ischemic damage to the optic nerve. Papilledema is caused by increased intracranial pressure and the pathological mechanism of disease involves disrupted axonal transport.[1–5]

Clinical background

Historical development

The quest to understanding the mechanisms behind axonal degeneration began in the mid-1800s with the works of Augustus Waller. In 1850, he demonstrated that axons could undergo a compartmentalized degenerative process. By transecting frog hypoglossal and glossopharyngeal nerves, he noted that the distal axonal fragments (those separated from the cell soma) underwent a "curdling" and disorganization, and in 1856 noted a similar observation following transection of the rabbit optic nerve.[6] This type of axonal degeneration has been termed "wallerian degeneration." Since this time, more and more evidence has shown that axons undergo a compartmentalized degenerative process, using mechanisms distinct from those causing the degeneration and death of the cell soma.[7] Clinically, this has arisen parallel to the perception that certain CNS diseases may be considered "axogenic" diseases, or arising primarily from injury to the neuronal axon, whereas others may be considered "somagenic" diseases, which, in contrast, arise primarily from injury to the cell soma.[8]

In neuro-ophthalmic disease, the RGC body and its axon are the main targets of pathology, and just as in the rest of the CNS, the concept of somagenic versus axogenic disease applies. Diseases primarily affecting the RGC layer (somagenic diseases) are usually retinopathies, and result from injuries including ischemia, excitotoxicity, autoimmune processes, thermal and photic injury, storage diseases, neoplastic processes, nutritional deprivation, and toxins.[1] Axogenic diseases of the optic nerve and the mechanisms underlying their pathophysiology are the subject of this chapter.

Key symptoms and signs

Diseases characterized by optic nerve axonal injury are associated with abnormal visual acuity, color vision, visual field, and optic nerve head color and morphology. Ischemic, traumatic, compressive, infiltrative, inflammatory, infectious, toxic, and nutritional optic neuropathies are often associated with decreased visual acuity early in the course of disease because of significant involvement of RGCs sending axons via the papillomacular bundle. Open-angle glaucoma, papilledema, and optic disk drusen affect the papillomacular bundle much later on, thereby not initially causing reduction in visual acuity. Similarly, color vision is variably affected. Color vision loss, which usually affects the red–green axis, occurs late in glaucomatous optic neuropathy.

Optic nerve excavation and pallor, which reflect the effect of chronic loss of RGC axons, are elements that can help distinguish the various optic neuropathies. Histologically, excavation represents loss of all tissue and thus leads to the creation of an empty space, or cup, surrounded by axon-containing tissue, called the rim. Pallor is caused by axonal loss, but occurs in the presence of remaining viable glial tissue. This glial tissue takes the place of the space that would

Box 42.1 *Clinical features of common axonal diseases of the retinal ganglion cell*

Disease	Clinical features	Genetics	Epidemiology	Treatment	Prognosis
Glaucoma	Progressive visual field defects, starting peripherally and eventually involving fixation (and, consequently, visual acuity) Normal color vision Characteristic disk cupping without signficant rim pallor Elevated intraocular pressure (IOP) frequently present	Family history important, and several known genes, e.g., myocilin (MYOC) for primary and juvenile open-angle glaucoma; optineurin (OPTN) for primary open-angle glaucoma; CYP1B1 for congenital glaucoma	Second leading cause of blindness in the world[2] Risk factors Age Race Family history Elevated IOP Myopia	Lowering of IOP	Many patients progress despite lowering of IOP
Optic neuritis	Decreased visual acuity and color vision acutely Pain on eye movements Visual field loss, often central Optic disk swollen in 30% of patients Disk pallor begins weeks after attack	Similar to those of multiple sclerosis (MS), e.g., HLA complex (HLA-DR2 haplotype)	15–20% of patients with MS present with optic neuritis initially Occurs in 50% of MS patients Risk factors Young adults Female > male MS	Intravenous methylprednisone Disease-modifying drugs for MS	Usually complete or almost complete recovery of vision (20/40 or better) May have residual color vision or visual field deficits
Papilledema	Bilateral optic disk swelling from increased intracranial pressure Transient visual obscurations Relatively preserved visual acuity, color vision and field acutely Late visual field changes: nasal depression, arcuate scotomas, concentric constriction of field	Dependent on disease causing elevated intracranial pressure	Idiopathic intracranial hypertension (obesity) Intracranial tumor Cerebral venous thrombosis	Removal of tumor Weight loss, acetazolamide, topiramate, lumbar peritoneal or ventriculoperitoneal shunting, optic nerve sheath fenestration	Prognosis dependent on severity and duration of intracranial hypertension Optic atrophy and arterial narrowing are late signs
Arteritic anterior ischemic optic neuropathy	Acute loss of vision Unilateral initially and, if untreated, bilateral in days to weeks Headache, jaw claudication, weight loss, neck and shoulder pain Scalp pain, myalgias, fever Optic disk edema	Sporadic	20 cases/100 000 persons over age of 50 annually Risk factors Women > men Mostly Caucasians Age 65 or older[113]	Intravenous followed by oral corticosteroids Temporal artery biopsy to confirm diagnosis	Profound visual loss: counting fingers to no light perception Rarely improvement in vision Corticosteroids prevent vision loss in second eye in most patients
Nonarteritic anterior ischemic optic neuropathy	Usually acutely decreased visual acuity Color vision relatively preserved Visual field defects, which are usually altitudinal Optic disk edema	Sporadic	2.3–10.2 cases/100 000/year over 50 years of age Mostly Caucasians[113] Risk factors Small cup-to-disk ratio Diabetes, hypertension. ? Nocturnal hypotension[113,115]	No proven treatment	Visual acuity from 20/20 to light perception 42% of patients improve by 3 lines or more Rarely recurs in same eye Increased risk of second eye involvement (up to 15% at 5 years)[115,116]
Compressive optic neuropathy	Slowly progressive painless decrease in visual acuity Decreased color vision Disk edema and/or atrophy Central or cecocentral field defects Also proptosis, strabismus, optociliary shunt vessels (meningiomas and gliomas)	Dependent on cause of disease	Risk factors Middle-aged female > male for meningiomas Neurofibromatosis 1 for gliomas Thyroid orbitopathy	Removal or reduction of compressive mass by surgery, radiotherapy, or chemotherapy	Usually slowly progressive decrease in vision unless treatment undertaken Gliomas may remain stable
Traumatic optic neuropathy	Acutely decreased visual acuity and color vision Optic nerve pallor develops after a few weeks	None	Up to 5% of indirect head injuries may have a traumatic optic neuropathy[114]	No treatment shown to be superior to observation alone	Spontaneous improvement may occur, but prognosis often poor, especially if no light perception at onset
Infiltrative optic neuropathy	Subacute, chronic decrease in visual acuity and color vision May present with headache Other cranial nerves may be affected Optic disk either normal or edematous	Dependent on cause of disease	Risk factors Granulomatous disease (e.g., sarcoidosis), neoplasms (leukemia, lymphomas, metastases), optic nerve glioma	Treatment of underlying disease	Dependent on disease process

Table 42.1 Disorders with disk excavation

Glaucoma
Compressive optic neuropathies (sometimes)
Methanol optic neuropathy
Arteritic anterior ischemic optic neuropathy
Shock optic neuropathy
Dominant optic neuropathy
Leber's hereditary optic neuropathy
Periventricular leukomalacia

otherwise increase the size of the excavation, producing instead a pale area. In glaucoma, the morphology of the excavation is almost pathognomonic, although other optic neuropathies may have significant excavation (Table 42.1). Pallor of the neuroretinal rim is eventually seen in most nonglaucomatous optic neuropathies. On the other hand, cupping in the absence of pallor is typical of glaucomatous optic neuropathy. This rule is not without exception, however, as end-stage glaucoma may be associated with a pale rim (see Chapter 44).

Visual field abnormalities help to differentiate the optic neuropathies. Optic neuropathies that are caused by more anterior or optic nerve head damage usually give rise to defects that follow the RGC axon distribution pattern in the retina, or nerve fiber bundle defects. Glaucoma is the best known of these, and typically causes arcuate scotomas, nasal steps, and temporal wedges. Other neuropathies which may cause similar types of visual field changes include papilledema and optic disk drusen. In the beginning stages, papilledema may give rise to an enlarged blind spot that is refractive (due to elevation of the peripapillary retina). This is followed by nasal steps, arcuate visual field defects, and concentric constriction of the visual field. Nonarteritic anterior ischemic optic neuropathy typically gives rise to altitudinal field defects, mostly inferiorly, which may also cross the horizontal meridian and involve fixation and are therefore not strictly nerve fiber bundle defects. Optic neuropathies due to axonal injury posterior to the optic nerve head, yet anterior to the chiasm, frequently produce central scotomas or diffuse visual field loss. Included in this category are optic neuritis, compressive, infiltrative, toxic and nutritional optic neuropathies, as well as Leber's hereditary optic neuropathy and autosomal-dominant optic neuropathy.[4]

Epidemiology

See Box 42.1.

Genetics

See Box 42.1.

Diagnostic workup

The diagnosis of many axonal injuries of the optic nerve can be made on the basis of neuro-ophthalmic history and examination. Neuroimaging is typically the first step when the diagnosis is not otherwise apparent, especially when there is optic nerve edema, pallor of the neuroretinal rim, central visual field loss, or visual field loss that respects the vertical meridian. Examination of the blood or cerebrospinal fluid and other imaging techniques are determined based on the specific clinical syndrome.

Differential diagnosis

See Box 42.1.

Treatment and prognosis

Treatment and prognosis depend on specifics of the optic neuropathies. There are no proven treatments for nonarteritic anterior ischemic optic neuropathy, congenital and hereditary optic neuropathies, most traumatic optic neuropathies, and compressive optic neuropathy other than decompression of the instigating mass. Lowering the intraocular pressure has been proven to decrease the rate of progression of glaucomatous optic neuropathy. Patients with optic neuritis will recover vision more quickly when treated with intravenous corticosteroids, but the final visual outcome is the same as with placebo. Intravenous and oral corticosteroids may stop progression or save the unaffected eye in arteritic anterior ischemic optic neuropathy.

Pathology

The mechanisms responsible for optic neuropathies reflect different types of axonal injury. Major underlying causes include ischemia, demyelination, inflammation, compression, transection, glaucoma, infiltration, and papilledema. Two or more of these factors may be involved in one optic nerve disease. For example, both demyelination and inflammation are implicated in the pathogenesis of optic neuritis or other active lesions in multiple sclerosis. In multiple sclerosis, demyelination is thought to lead to loss of axonal trophic support, whereas inflammatory mechanisms may lead to either direct (immunologic attack) or indirect (through cytokines and proteolytic enzymes) axonal injury.[9] In glaucoma, increased intraocular pressure is thought to lead to mechanical deformations of axons, disrupted axonal transport, and microvacular ischemia.[10]

On a microscopic level, mitochondrial dysfunction, disruption of axonal conduction, disruption of axonal transport, and axonal transection can each result from the different types of axonal injury and contribute to the pathogenesis of the various optic neuropathies (Box 42.2). Ultrastructurally, the first sign of axotomy-induced axonal degeneration includes a rounding and swelling of the axolemma, occurring in the first 12–24 hours following injury in rats and up to 7 days for humans.[11] This is followed by calcium entry into the cell, the subsequent activation of calcium-dependent proteases (calpains), and activation of the ubiquitin proteosome system. These processes ultimately lead to the degradation of microtubules and neurofilaments, contributing to axonal disassembly. Wallerian degeneration demonstrates multiple dense bodies, neuroaxonal spheroids, and retraction balls at the sites of axonal transaction.[12] Blockage of extracellular calcium channels or inhibition of the ubiquitin-proteasome system is enough to delay the

Box 42.2 Pathogenesis of common axonal diseases of retinal ganglion cells

Optic neuropathy	Pathogenesis
Glaucoma	Mechanical, ischemic, or inflammatory injury of axons at the optic disk Interruption of axonal transport Oxidative free radical generation[1]
Optic neuritis	Demyelination of optic nerve (loss of trophic factors) Immune-mediated axonal damage Axonal conduction block Mitochondrial dysfunction and energy crisis Axonal transection and loss[1,9]
Papilledema	Intra-axonal edema causing abnormalities in axonal transport[1]
Arteritic anterior ischemic optic neuropathy	Occlusion of posterior ciliary arteries and infarction of optic nerve head [4]
Nonarteritic anterior ischemic optic neuropathy	Decreased perfusion
Compressive optic neuropathies	Conduction block, ischemia, demyelination, and axonal transection
Traumatic optic neuropathies	Transection, avulsion, hemorrhage (direct injury) or stretch, compression (indirect injury)
Infiltrative optic neuropathies	Infiltration, compression of optic nerve

processes of axonal degeneration.[13,14] The importance of calcium in mediating axonal degeneration cannot be overemphasized; increasing extracellular calcium concentrations alone (in the absence of axotomy) is sufficient to cause axonal degeneration in mouse dorsal root ganglia cultures.[13]

The pathological end-stage of axonal optic neuropathy is optic atrophy, and this is discussed in Chapter 44, as well as in the pathophysiology section, below.

Etiology

A variety of risk factors have been associated with optic nerve axonal injuries, including age, sex, race, family history, ocular morphology, systemic disease, nutritional factors, exposure to toxins, and possibly other environmental factors (see Box 42.2 and individual chapters for specifics). For example, optic neuritis and Leber's hereditary optic neuropathy are more common in the young, whereas glaucoma and ischemic optic neuropathy are more prevalent in older individuals. Optic neuritis and arteritic anterior ischemic optic neuropathy are more common in women and Leber's hereditary optic neuropathy is more common in men. Examples of ocular morphology risk factors include a greater incidence of glaucoma in myopic individuals and of nonarteritic anterior ischemic optic neuropathy in those with small, crowded optic nerve heads. Systemic disease risk factors include neurofibromatosis (optic gliomas), thyroid disease (compressive optic neuropathy), and many more. Genetic risk factors are profound in hereditary optic neuropathies, e.g., Leber's hereditary optic neuropathy and dominant optic atrophy, but play an important role in glaucoma, optic neuritis, and disk drusen. Some studies have demonstrated that genetics may also play a role in the ischemic optic neuropathies.

Pathophysiology

As discussed above, the optic neuropathies generally arise from some form of injury to retinal ganglion axons. In some cases, the site of injury is obvious, e.g., traumatic optic neuropathies. In other cases, e.g., glaucoma, there is less direct evidence that the initial sites of injury are the RGC axons within the optic nerve head. Several studies have demonstrated that early injury occurs at the lamina cribrosa in glaucoma.[15–18] Additional findings, including focal notching of the disk[19] and splinter hemorrhages,[20,21] have also helped to pinpoint the optic disk as the initial site of injury. More recent evidence from the DBA/2J mouse model of glaucoma confirms the axonal locus of injury.[22,23]

The effects of the axonal injury are numerous, not only on the optic nerve and RGCs, but also on other cells. The concept of axonal degeneration has recently been reviewed[24] (Figure 42.1). This section discusses some of the major consequences of axonal injury, focusing on the following: (1) effects on the RGC body; (2) wallerian degeneration of the axon distal to the injury site; (3) retrograde degeneration proximal to the injury site; (4) effects on other neurons; and (5) effects on nonneuronal cells. There are numerous other effects, e.g., excitability, axonal conduction, and particularly changes in the dendritic arborization,[25–27] which are areas of active study.

Effects on the retinal ganglion cell body

Axonal injury is the first step to occur in most of the optic neuropathies, and leads to RGC death.[28] Depending on the animal species affected, the distance of injury from the RGC, and the cell body size, up to 70% of ganglion cells may survive following axonal injury.[1] Studies of the time course of RGC death in rodents after optic nerve injury demonstrate a partial (20–40%) loss within the first 3–7 days, the remainder (50–90%) taking weeks to sometimes months.[29–34] Nonhuman primate RGCs die approximately 4–6 weeks after axotomy.[28,35] In humans there is sparing of some ipsilateral temporal ganglion cells 35 days after transection of the optic tract,[36] and even in absolute glaucoma some RGCs seem to survive.[37] In lower animals, e.g., goldfish and frogs, ganglion cells do not die, but hypertrophy and regenerate axons within 1–2 months.[38–40] The fact that ganglion cell death is not a necessary consequence of axotomy suggests that there are regulatory mechanisms underlying RGC survival after axonal injury.

Axonal injury following axotomy, elevated eye pressure, ocular ischemia, optic nerve crush injury, and occurring subsequent to the destruction of postsynaptic target cells has been shown to lead to RGC death through apoptosis.[41–43] As with apoptosis in other tissues, there is chromatin condensa-

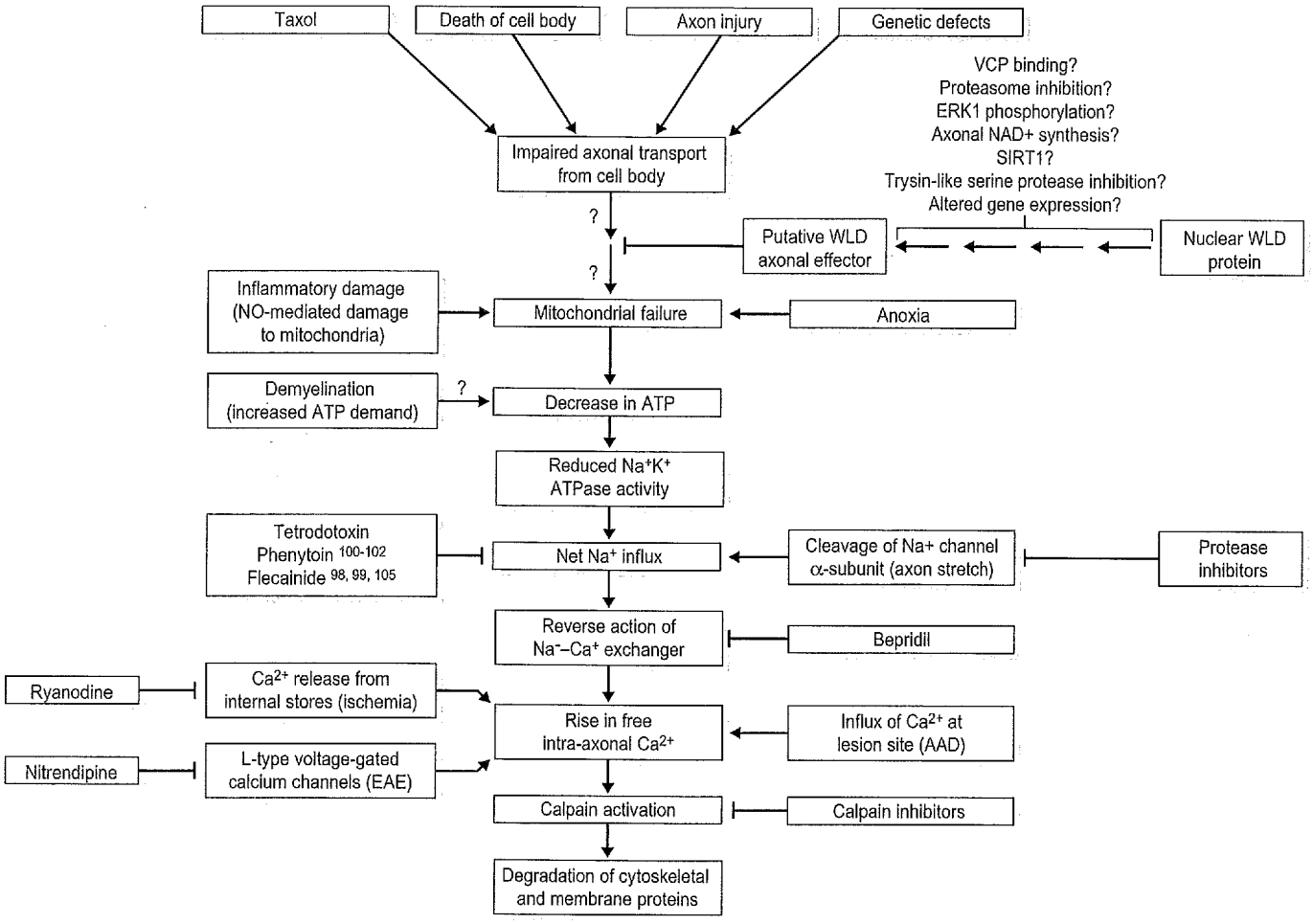

Figure 42.1 Example of some of the multiple pathways activated by axonal injury. (Redrawn from Coleman M. Axon degeneration mechanisms: commonality amid diversity. Nat Rev Neurosci 2005;6:889–898.)

tion, shrinkage, and phagocytosis by surrounding cells, and it appears to be predominantly mediated by the bcl-2 family of proteins, containing proapoptotic members Bax, Bid, and Bad and antiaptoptotic members Bcl-2 and Bcl-X, and the cysteine protease family of caspases.[44] Though the exact signaling process leading from nerve fiber layer injury to RGC apoptosis has not been elucidated, many molecular mechanisms are involved. For example, deprivation of neurotrophic factors from the target or other tissues, excitotoxicity from physiological or pathological levels of glutamate, free radical formation, increases in intra-axonal Ca^{2+}, induction of neuronal endopeptidases, accumulation of excess retrogradely transported macromolecules, and induction of p38 MAP kinase and other signaling molecules may stimulate the apoptotic pathway.[29,45–54]

Of these, the most well-studied mechanism to date involves loss of neurotrophin support from target cells. It has long been known that neurotrophic factors are essential elements in neuronal development. During development, neurons are present in excess numbers. According to the neurotrophic factor hypothesis, developing neurons compete for a limited number of target-derived neurotrophic factors that are required for survival and differentiaion. Lack of appropriate target cell contacts leads to neurotrophin deprivation in the developing neuron, with subsequent axonal pruning and death of the cell soma by apoptosis.[55,56] Target cell integrity and neurotrophin support are also essential elements in maintaining the survival of adult RGCs.[57–59] Following nerve fiber layer injury, disruption of orthograde and retrograde axonal transport of neurotrophins can occur and this may ultimately lead to RGC neurotrophin deprivation and subsequent apoptosis. This has mostly been demonstrated with intraocular pressure elevation in primate and rat models.[60–65] Support for the role of neurotrophin dependence particularly comes from experiments using identified neurotrophic factors to rescue axotomized neurons. Purified neonatal RGCs (which are axotomized during dissociation) can be kept alive for significant periods with a cocktail of factors, including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor, forskolin, and insulin.[66] Intraocular administration of certain neurotrophins (e.g., BDNF) delays RGC death after axotomy in adult rats[33,49] and cats,[67] and in an experimental model of glaucoma.[68] Gene delivery of BDNF to the retina or to the RGC itself also increases survival in experimental glaucoma,[69,70] as it does inhibition of apoptosis.[71]

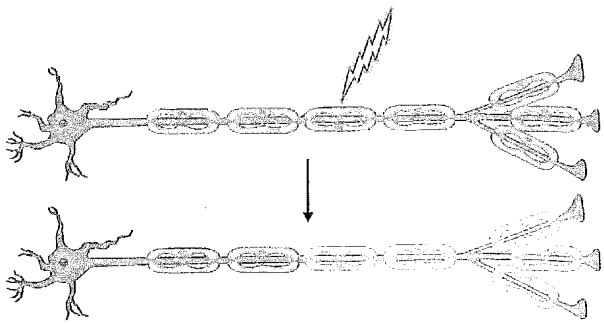

Figure 42.2 Optic nerve injury induces intra-axonal and extra-axonal pathophysiology. An early event is wallerian degeneration, in which the distal axon degenerates. (Redrawn from Di Polo A. Mechansms of neural injury in glaucoma. In: Levin LA, Weinreb RN, Di Polo A (eds) Neuroprotection for Glaucoma: A Pocket Guide. New York: Ethis, 2007.)

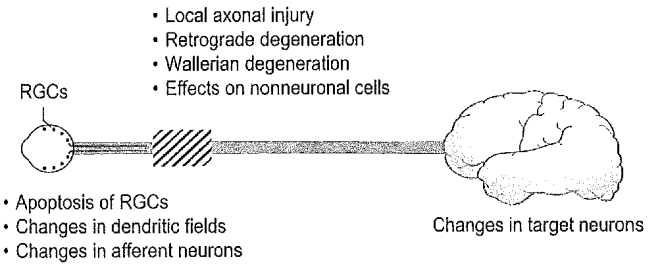

Figure 42.3 Axonal injury affects the retinal ganglion cell's axon, dendrites, and soma, other neurons, and nonneuronal cells in the optic nerve and retina.

There are likely other signals for RGC death besides neurotrophin deprivation. RGCs maintain viability for long periods of time when there is decreased axonal transport from compressive optic neuropathy or papilledema. Retrograde axonal transport is rapid, and the subacute time course by which RGCs die after axonal injury does not reflect the time course of interrupted retrograde axonal transport. RGC axotomy induces changes in responsiveness to neurotrophins independent of neurotrophin deprivation.[72] Finally, removal of the RGC axonal target, and therefore, target-derived factors, causes very slow RGC death.[57,73] These findings suggest that axotomy can signal changes at the cell body independent of neurotrophin deprivation. For example, an elevation in intracellular levels of the reactive oxygen species superoxide can occur independent of neurotrophin deprivation, and is necessary and sufficient for RGC death after axotomy.[74]

Wallerian (anterograde) and retrograde degeneration

Wallerian degeneration

Axonal injury in the CNS and PNS arising from traumatic, metabolic, toxic, inflammatory, and hereditary causes often results in a form of secondary axon pathology termed wallerian degeneration[6] (see section on historical development, above). Focal injury to the axon leads to an orderly disassembly of the distal axonal stump in the hours to days following injury (Figure 42.2). At the cellular level, there is initial disassembly of the myelin sheath, followed by swelling of the axolemma, disorganization of neurofilaments and microtubules, and mitochondrial swelling. The remaining axonal fragments then undergo phagocytosis by glial cells and macrophages, followed by apoptosis of surrounding oligodendrocytes in the CNS.[11]

The directionality of wallerian degeneration was explored by Beirowski et al,[75] who showed that, in mouse peripheral nerves, wallerian degeneration proceeds asynchronously (at different rates between different axons) and either anterogradely or retrogradely depending on whether it was a transection or crush injury that was incurred, respectively.[75] Studies performed on dorsal root ganglion nerves had shown similar results, demonstrating that CNS transection injury causes anterograde degeneration. Wallerian degeneration may occur in a variety of diseases, including demyelinating diseases such as multiple sclerosis and Guillain–Barré, as well as following neurovascular insults, and neurodegenerative and infectious processes.[12] As would be expected, wallerian degeneration is seen in optic nerve diseases.[22,76–82]

Dying-back degeneration

Another well-characterized type of axonal degeneration occurs by a process called dying back. As opposed to wallerian degeneration, which is thought to occur due to localized injury, dying-back degeneration occurs following a chronic and more generalized form of injury to the axon. It is a slow process, occurring over weeks to months, and involves degeneration of the axon from the synaptic end towards the cell soma in a retrograde fashion. It is known to occur in peripheral neuropathies, but in recent years evidence has accrued that it also is important for the pathophysiology of CNS degenerations (e.g., Alzheimer's disease and Parkinson's disease).[7] Presumably a chronic optic nerve injury from a toxin, mitochondrial dysfunction, or nutritional deficiency could cause a parallel pathology to RGC axons.

Acute axonal degeneration

Though wallerian degeneration is the main form of axonal degeneration following axotomy, another degenerative process may occur in the acute stages of transection injury (beginning 20 minutes postinjury and lasting 5 minutes). Using in vivo time lapse imaging studies, Kerschensteiner and colleagues[82] demonstrated that a process which they referred to as acute axonal degeneration (AAD) may occur prior to wallerian degeneration following dorsal root transection in mice. AAD is thought to occur in the minutes following axotomy, causing axonal fragmentation in a bidirectional fashion in both the proximal and distal axonal stumps. This is thought to lead to retraction of the proximal axonal stump and subsequent wallerian degeneration of the distal end.[82,83]

Axonal degeneration is independent of soma degeneration

The mechanisms responsible for axonal degeneration are distinct from those causing apoptosis (Figure 42.3). Axonal degeneration often involves the calpains and/or the ubiquitin-proteosome system, unlike caspases in apoptosis.

Figure 42.4 Axonal injury when apoptosis is blocked with bax knockout. The soma and proximal axon are spared (green), but the distal axon undergoes wallerian degeneration (red).

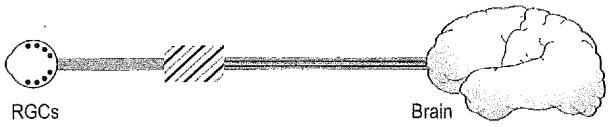

Figure 42.5 Axonal injury when wallerian degeneration is blocked in the Wld^S mutant. The distal axon is spared (green), but the proximal axon dies back and the soma undergoes apoptosis (red).

In recent years the distinction between apoptosis and axonal death has become clearer. It is now evident that, even though axonal death usually occurs following apoptosis (and vice versa), apoptosis is not a necessary requirement for axonal death. Axonal degeneration does not appear to occur due to a process of "starvation" from the cell soma, but rather seems capable of undergoing its own autonomous death process.

Blocking apoptosis

The DBA/2J Bax knockout mouse is just one of the experimental models which have helped to demonstrate this. DBA/2J mice develop elevated intraocular pressure and glaucoma as they age. DBA/2J mice crossed with mice with knockout alleles for Bax undergo axonal degeneration, but are protected from cell body apoptosis[84] (Figure 42.4). These results demonstrate that axonal degeneration is distinct from apoptosis in glaucoma and that axonal death can occur in the absence of death of the cell soma.

Other clues have emerged from bcl-2 transgenic mice. Axotomy of nerves from mice containing the human bcl-2 transgene undergo axonal degeneration at a rate comparable to wild-type mice, but are protected from apoptosis of the cell soma. This demonstrates the importance of bcl-2 in preventing cell body death but its failure to protect from axonal degeneration.[85] The pmn mouse, a mouse model of a motor neuropathy, is another example of the concept that axonal degeneration and death of the cell soma are distinct compartmentalized processes. Overexpression of the bcl-2 gene or inactivation of the bax gene in pmn mice prevents the death of the motor neuron cell bodies, but does nothing to prevent axonal degeneration.[86] These mice continue to develop weakness and eventually die at a normal rate, despite protection from neuronal apoptosis.

Wallerian degeneration slow (Wld^S) mutants

Perhaps the most impressive evidence that axonal degeneration may occur independently from death of the cell soma was demonstrated using the wallerian degeneration slow (Wld^S) strain of mice. Wld^S mice have an autosomal-dominant 85-kb tandem triplication mutation on chromosome 4 which confers delayed wallerian degeneration in both the PNS and CNS. In this strain of mice, axonal degeneration following injury or neurotrophin deprivation is delayed severalfold compared to wild-type mice, taking place several weeks after injury compared to only hours or days after. Remarkably, though axonal degeneration is delayed in these mice, death of the cell soma proceeds at a rate comparable to that of wild-type mice. That is, following injury, the process of apoptosis is not slowed or delayed in Wld^S mice.[87,88] Axonal loss is slowed in glaucoma models in mice[22] and rats[89] when on a Wld^S background (Figure 42.5). These results provide distinct and parallel evidence that axonal degeneration and apoptosis likely occur by very different and autonomous molecular mechanisms.

The genetic mutation responsible for the delayed Wallerian degeneration phenotype is an in-frame fusion of the N-terminal 70 amino acids of the E4 ubiquitin ligase Ube4b, an 18-amino-acid linker, and the full-length nicotinamide mononucleotide adenylyl transferase 1 (Nmnat1), involved with NAD^+ synthesis.[90,91] All parts are necessary for in vivo protection of axons from wallerian degeneration, and it is likely that direction of the fusion protein to a specific subcellular compartment is necessary for full effects.[92–94] How this fusion protein blocks wallerian degeneration is an area of active research.[93,95,96]

Effects of axonal injury on other neurons

Axonal injury may also lead to the secondary degeneration of surrounding axons (which were uninjured by the primary insult). Partial optic nerve crush and transection injury models have demonstrated initial rapid injury of directly damaged axons, followed weeks or months later by degeneration of axons in adjacent areas.[97–99] This was first demonstrated following traumatic injury to the CNS[100] and is proposed to be a possible cause of continued RGC loss in optic neuropathies, particularly glaucoma, despite intraocular pressure control. Regardless of the type of injury, this secondary degeneration is believed to occur through a variety of mechanisms, including excitatory neurotransmitter (glutamate) or oxygen free release by primarily injured axons, or by changes in extracellular ion concentrations, particularly potassium levels.[51,97,101] Support for glutamate involvement stems from experiments using MK-801 (an *N*-methyl-d-aspartate (NMDA) receptor antagonist) following optic nerve crush injury, which has been shown to attenuate secondary degeneration.[102]

Finally, the impact of axonal degeneration is not only visible in close proximity to the initial lesion, but may also extend to target cells, located much further away. For example, the vast majority of RGCs establish terminal synapses in the lateral geniculate nucleus (LGN), and in several models of glaucoma, it has been shown that atrophy of cells in the LGN correlates with severity of RGC axon loss and of intraocular pressure (see Chapter 26).

Effects of axonal injury on nonneural cells

Though most work has focused on axonal injury and the subsequent effects on RGC death and/or survival, axonal disruption may also have consequences on surrounding retinal cells. Following optic nerve crush or spinal cord injury, for example, oligodendrocytes undergo apoptosis.

The loss of axonal contact and the decrease in neurotrophic factors are believed to lead to the initiation of the apoptotic program or of an atrophy-like resting state in oligodendrocytes following wallerian degeneration.[103–106] Members of the tumor necrosis factor cytokine receptor superfamily and other molecules are involved in nonneuronal cell death after axonal injury.[107,108]

Other cells which are affected by axonal injury in the CNS include the resident microglia. These phagocytic cells increase in size and number and undergo activation in response to wallerian degeneration, occurring several days later than the macrophage response in the PNS.[11,109] Though they undergo activation following axonal injury,[110] their phagocytic response nevertheless is limited and they incompletely clear myelin debris in the CNS.[111] This is in contrast to PNS injury, where macrophages are actively recruited from the circulation and phagocytose myelin debris in an opsonin-dependent manner.[112] Unlike in the PNS, there is a less profound influx of macrophages from the circulation during CNS injury.[11]

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A complete list of chapter references is available online at www.expertconsult.com. See inside cover for registration details.

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Regulation of Neurotrophin-Induced Axonal Responses via Rho GTPases

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ABSTRACT

Nerve growth factor (NGF) and related neurotrophins induce differential axon growth patterns from embryonic sensory neurons (Lentz et al. [1999] J. Neurosci. 19:1038–1048; Ulupinar et al. [2000a] J. Comp. Neurol 425:622–630). In wholemount explant cultures of embryonic rat trigeminal ganglion and brainstem or in dissociated cell cultures of the trigeminal ganglion, exogenous supply of NGF leads to axonal elongation, whereas neurotrophin-3 (NT-3) treatment leads to short branching and arborization (Ulupinar et al. [2000a] J. Comp. Neurol. 425:622–630). Axonal responses to neurotrophins might be mediated via the Rho GTPases. To investigate this possibility, we prepared wholemount trigeminal pathway cultures from E15 rats. We infected the ganglia with recombinant vaccinia viruses that express GFP-tagged dominant negative Rac, Rho, or constitutively active Rac or treated the cultures with lysophosphatitic acid (LPA) to activate Rho. We then examined axonal responses to NGF by use of the lipophilic tracer DiI. Rac activity induced longer axonal growth from the central trigeminal tract, whereas the dominant negative construct of Rac eliminated NGF-induced axon outgrowth. Rho activity also significantly reduced, and the Rho dominant negative construct increased, axon growth from the trigeminal tract. Similar alterations in axonal responses to NT-3 and brain-derived neurotrophic factor were also noted. Our results demonstrate that Rho GTPases play a major role in neurotrophin-induced axonal differentiation of embryonic trigeminal axons. J. Comp. Neurol. 438:377–387, 2001.

Indexing terms: Rac; Rho; trigeminal axon growth; NGF; NT-3; BDNF; viral vectors

Primary sensory neurons of the dorsal root and trigeminal (TG) ganglia depend on target-released neurotrophins for survival and express specific Trk receptors (McMahon et al., 1994; Wright and Snider, 1995; Conover and Yancopoulos, 1997; Davies, 1997, Davies,1998; Enokido et al., 1999; Huang et al., 1999b). Recent studies also suggest that neurotrophins play a major role in axonal differentiation (Hoyle et al., 1993; Lentz et al., 1999; Ulupinar et al., 2000a; see also Gallo and Letourneau, 2000, for a review). During axonal development, elongation and retraction of growth cones are all related to the arrangement of actin cytoskeleton (for reviews, see Lin et al., 1994; Hall, 1998; Gallo and Letourneau, 2000). The Rho family of GTPases is known to play a major role in this process (for reviews, see Luo et al., 1997; Tapon and Hall, 1997; Aspenstrom, 1999; Mueller, 1999; Song and Poo, 1999; Bishop and Hall, 2000).

Members of the family (Cdc42, Rho, and Rac) are either found in the GTP-bound active state or the GDP-bound inactive state (for reviews, see Van Aelst and D'Souza-Schorey, 1997; Mackay and Hall, 1998; Hall 1999; Kjoller and Hall 1999; Bishop and Hall, 2000). Relative abundance of active Rho and Rac might regulate growth cone behavior during axon navigation (Mueller, 1999). Dominant negative and constitutively active versions of Rho GTPases have been used to study their role in axonal arborization (Threadgill et al., 1997; Nakayama et al., 2000), dendritic growth (Threadgill et al., 1997; Ruchoeft et al., 1999; Nakayama et al., 2000; Li et al., 2000), neuronal remodeling (Luo et al., 1996; Ruchoeft et al., 1999; Li et al., 2000), and growth cone motility (Jin and Strittmatter, 1997; Kozma et al., 1997; Ruckhoeft et al., 1999; Kuhn et al., 2000; Nakayama et al., 2000: Wahl et al., 2000). Dominant negative proteins bind irreversibly to the specific GEF molecules, which mediate the exchange of GDP to GTP and compete for the activation of endogenous

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small GTPases inside the cell. Thus, expression of dominant negative constructs mimics downregulation of effector molecules for the particular Rho GTPase. Constitutively active constructs are unresponsive to regulatory proteins and are in the activated state at all times.

Although it has also been suggested that neurotrophins regulate the neuronal cytoskeleton (Gallo and Letourneau, 2000), the involvement of Rho GTPases in neurotrophin-mediated axonal changes is largely unknown. To elucidate this issue, we investigated the role of Rac and Rho in axonal responses of central trigeminal axons to NGF, NT-3 and BDNF.

The embryonic rodent trigeminal pathway can be isolated as an intact wholemount preparation. Exogenous application of NGF and NT-3 to these cultures has differential effects on growth parameters of central trigeminal axons in the brainstem. In the presence of NGF, TG axons leave the central trigeminal tract and extend into the surrounding brainstem tissue without branching, whereas NT-3 promotes precocious arborization of tract axons (Ulupinar et al., 2000a). Localized application of neurotrophins also yields similar results (Erzurumlu et al., 2000). In the present study, we prepared TG-brainstem wholemount cultures and infected the ganglion with the recombinant vaccinia viruses that express the dominant negative and constitutively active Rac or Rho GTPases. We then tested the effects of neurotrophins on axon outgrowth from the central trigeminal tract.

MATERIALS AND METHODS

Preparation of trigeminal pathway explant cultures

Embryos from 15-day pregnant Sprague-Dawley rats (day of sperm positivity E0) were removed by cesarean section following euthanasia of the dam. All the experimental procedures were conducted according to NIH and Institutional IACUC guidelines. Trigeminal pathway explants were dissected in ice-cold Gey's balanced salt solution (GIBCO, Gaithersburg, MD) suplemented with D-galactose (6.4 mg/l; Sigma, St. Louis, MO). These explants included the left and right TG and the brainstem from the pontine flexure to the upper cervical levels where the central trigeminal tract lies (Fig. 1A). Wholemount explants were then cultured on microporous Millicell membranes (Millipore, Bedford, MA) and grown in serum-free culture medium (SFM) at 33°C in a humidified CO_2 incubator (Ulupinar et al., 2000a).

Abbreviations

β-gal	β-galactosidase
BSA	bovine serum albumin
BDNF	brain-derived neurotrophic factor
DAB	3,3′-diaminobenzidine tetrahydrochloride
DiI	1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate
DRG	dorsal root ganglion
Dtr	descending trigeminal tract
E	embryonic day
GEF	guanosine nucleotide exchange factor
GFP	green fluorescent protein
GDP	guanosine diphosphate
GTP	guanosine triphosphate
LPA	lysophosphatitic acid
NGF	nerve growth factor
NT-3	neurotrophin-3
NT-3	neurotrophin-3
PB	phosphate buffer
PBS	phosphate-buffered saline
RacAC	constitutively active Rac (RacV12)
RacDN	dominant negative Rac (RacN17)
RhoDN	dominant negative Rho (RhoN19)
SFM	serum-free culture medium
TG	trigeminal ganglion
Trk	receptor tyrosine kinase
WP	whisker pad
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Viral infection and neurotrophin treatment

After 24 hours in culture, TG on one side of each wholemount explant was infected with recombinant vaccinia viruses (gift of H. Cline), which express GFP-tagged dominant negative (DN) Rho (RhoN19), Rac (RacN17), or constitutively active (AC) Rac (RacV12). The construction and use of these viral vectors have been described in detail by Li et al. (2000). TG on the other side was infected with a GFP-expressing viral construct as a control. TG neurons were infected with high-titer virus (over 10^6 plaque-forming units [pfu]), and infections were restricted to the ganglion with the help of a Hamilton microliter syringe mounted to a micromanipulator (Fig. 1A). Fast green dye (1%) was used with the viral solution to visualize the injection site. We did not have viral vectors expressing RhoAC. Instead, we added lysophosphatidic acid (LPA; 10μM, Sigma), which is known to activate Rho in neuronal cells (Ridley and Hall, 1992; Jalink et al., 1994). Following viral infections, cultures were transferred to SFM supplemented with NGF 2.5s (50 ng/ml; Collaborative Biomedical Products, Bedford, MA), NT-3, or BDNF (50 ng/ml; Regeneron Pharmaceuticals, Tarrytown, NY) and maintained in vitro for an additional 3 days. GFP expression in the TG was observed after 24 hours under epifluorescence by using a fluorescence isothiocyanate (FITC) filter set. In all cases GFP expression was localized to the ganglion with no or minimal spread to the surrounding brainstem tissue (Fig. 1B). GFP expression within the TG was considered a general indicator of viral infection.

In a separate series of cultures, blue and white sepharose beads (200 μm diameter; gift of K. Muneoka) were washed twice with PBS (pH 7.4), air-dried, and soaked in NGF, NT-3, BDNF (100 ng/μl), or bovine serum albumin (BSA; Sigma, 100 ng/μl) overnight at 4°C. A single bead was implanted along the lateral side of the descending trigeminal tract (Fig. 1C). Viral infections were done 24 hours after bead implantation, and wholemounts were kept 3 more days in culture. In each culture, a neurotrophin-loaded bead (blue) was implanted on one side and a BSA-loaded bead (white) on the other side as a control. Bead experiments were carried out to specifically test the localized effects of neurotrophins on trigeminal axon growth patterns, and their regulation by Rho family of GTPases.

Immunohistochemistry and histochemistry

Two of the viral constructs we used (RacV12 and RacN17) were myc-tagged at the amino (N) terminal; the RhoN19 construct expressed β-galactosidase (β-gal) from an independent promoter, and the control viral vector expressed GFP/β-gal fusion protein. Therefore, we performed c-myc immunohistochemistry for RacN17 and RacV12, and β-gal histochemistry for RhoN19 and control

GFP viral constructs to further verify viral gene expression in infected TG.

For immunohistochemistry, 20-μm-thick frozen sections taken from the infected explant cultures were first preincubated in a blocking solution containing 0.3% Triton X-100 and 5% horse serum in phosphate buffer (PB, 0.1 M, pH 7.4) for 1 hour followed by overnight incubation in primary antibody solution (monoclonal anti-c-myc, 1:100; Oncogene, Cambridge, MA) in blocking solution. After several rinses in PB, sections were incubated in Texas Red-conjugated horse anti-mouse antibody (1:100; Jackson ImmunoResearch Laboratories, West Grove, PA) in blocking solution for 45 minutes. For β-gal staining, frozen sections through the RhoN19-infected TG were rinsed in PB and processed by using the β-gal Staining Kit (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol.

To further verify that the infected TG explants contained cells that express high-affinity neurotrophin receptors we used TrkA immunohistochemistry. Frozen sections through the virally infected TG explants were preincubated in PB with 10% goat serum and 0.3% Triton X-100 for 45 minutes and then overnight in TrkA antibody (gift of Dr. L. Reichardt) diluted at 1:2500 in blocking solution. After several rinses in PB, the sections were incubated in CY3-goat anti-rabbit secondary antibody (1:200 dilution in blocking solution; Chemicon, Temecula, CA) for 2 hours. For all immunohistochemical procedures, control sections were treated in the same manner, omitting the primary antibody.

DiI labeling

After 3 days in culture, wholemounts were fixed with 4% paraformaldehyde in PBS) (pH 7.4, 0.1 M), and small crystals of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, Eugene, OR) were inserted into the ganglion (Fig. 1C) to visualize central trigeminal axons in the brainstem. Cultures were kept at 33°C for 7–10 days for the lipophilic tracer to diffuse along the entire length of the trigeminal tract. DiI-labeled axons in selected cultures were later photoconverted in 0.15% DAB in 0.1 M Tris buffer (pH 8.2; Sandell and Masland, 1988). All the photographic documentation presented in this study was done by the use of a CoolSNAP digital camera attached to a Nikon Microphot-SA or a Nikon Diaphot inverted microscope. Digital images were transferred to a power PC, and the contrast and brightness of the images were adjusted by using the Adobe Photoshop program. In the preparation of the figures the right (control) sides of the explants were inverted to align

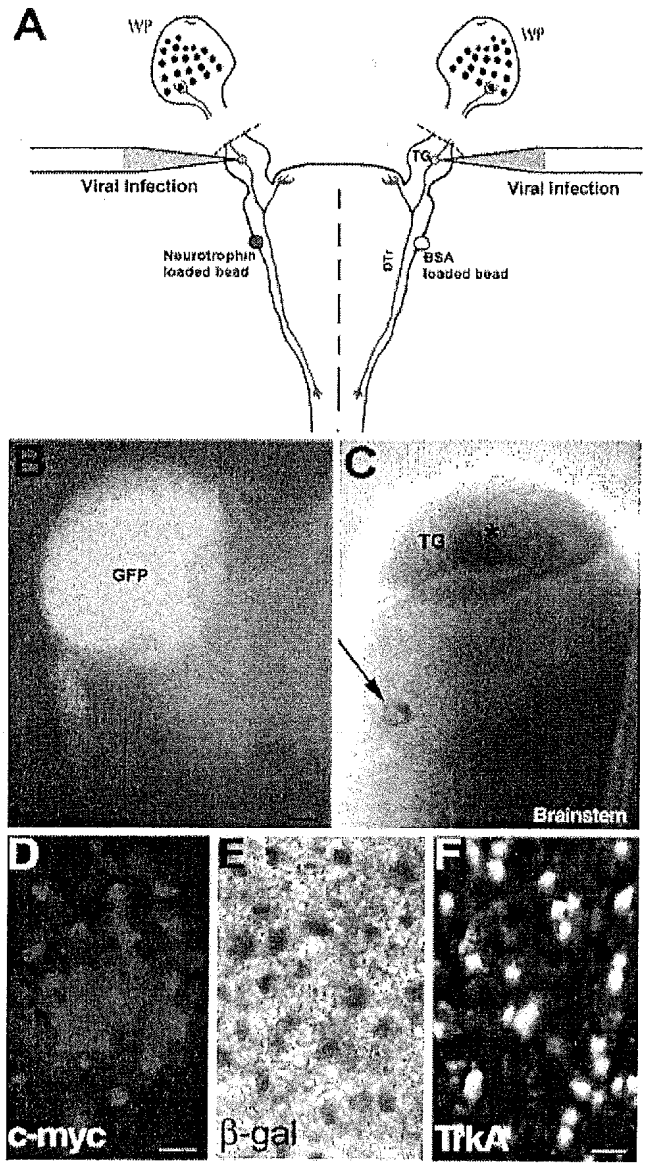

Fig. 1. Schematic diagram of the wholemount explant cultures and experimental procedures. **A:** The trigeminal pathway between the whiskerpad (WP) and the brainstem is illustrated. In the cultures used, the whiskerpad portion of the pathway was left out (dashed lines). The TG was then infected with GFP-tagged viral vectors. In each culture, one side was infected with control viral construct (GFP expression only) and the other side with a viral construct with GFP plus GTPase expression construct. These cultures were then maintained in either SFM or in medium supplemented with neurotrophins. In some of the cultures a color-coded (blue) bead soaked with a specific neurotrophin was implanted along the lateral side of the descending trigeminal tract (Dtr) on one side and a different colored (white), control, BSA-soaked bead was implanted in a similar position on the other side. Bead implanted cultures were maintained in SFM. **B:** Photomicrograph of a left half of the trigeminal pathway wholemount illustrating GFP expression restricted only to the trigeminal ganglion. **C:** Photomicrograph showing the trigeminal ganglion with a DiI crystal (asterisk) and a bead (arrow) placed along the lateral portion of the trigeminal tract. In B and C, and all the ensuing photomicrographs, rostral is up and medial is to the left. **D:** c-myc immunohistochemistry in RacV12 infected TG sections. Both RacV12 (RacAC) and RacN17 (RacDN) viral constructs show c-myc positive immunohistochemistry. **E:** β-Gal staining in RhoN19-infected TG sections. Both RhoN19 (RhoDN) and GFP (control) viral constructs lead to β-gal expression in TG cells (blue cells). **F:** Photomicrograph showing TrkA-positive cells in a section through a viral infected TG. *, site of DiI labeling; black arrow, site of neurotrophin-loaded bead. Scale bars = 200 μm for B and C; 30 μm for D–F.

the right and the left trigeminal tract in comparable positions, and no other alterations were made in the images.

Quantification of axon growth and statistical analysis

The results of each case (NGF [50 ng/ml], RacAC, RacDN, RhoDN, LPA, and NGF [100 ng/ml]) were captured with 10× magnification and printed on a full page. A transparent sheet with 50 × 50-μm grids was placed on top of the pictures, and the central trigeminal tract was labeled and accepted as zero reference point. For each case a constant length of the central trigeminal tract was used for measurements. Each grid containing an axon was counted as one. Both lateral and medial axon outgrowth was quantified. The number of grids containing axons for each 50-μm column was counted. The average and standard deviation of each 50-μm columnar distribution for each case were calculated with the help of the Microsoft Excel program. The result was plotted as the number of grids containing axons versus the length in consecutive 50-μm increments for both lateral and medial directions. For statistical evaluation, two pairs of metrics were used: mean length of axons and mean axon density in medial and lateral directions. Statistical comparisons were then made by using the one-tailed t-test. Before the application of the proper t-statistic, an F-test was used to detect any difference in the variance of experimental conditions (Montgomery, 1991). Error bars in the graphs represent one standard deviation.

RESULTS

Effects of NGF on central trigeminal axon growth patterns

The experimental culture model used in the present study is illustrated in Figure 1A. The wholemount trigeminal pathway with both the left and right TG intact allowed us to perform a given experimental procedure on one side and the control procedure on the other side in most experiments. Viral infection within the TG could be detected under epifluorescence due to the presence of GFP (Fig. 1B). In local neurotrophin application experiments, color-coded neurotrophin or carrier solution-loaded control beads were easily identified under microscopic examination (Fig. 1C). The viral constructs we used had a myc tag or β-gal expression in addition to GFP. Immunostaining for c-myc (Fig. 1D) or histochemical staining for β-gal (Fig. 1E) further confirmed the expression of gene products within the TG. In addition, immunostaining for the high-affinity NGF receptor TrkA also indicated that TG cells were not affected adversely by the viral infection, and small-diameter neurons expressed TrkA (Fig. 1F).

During normal development, central trigeminal axons grow along a restricted pathway in the lateral brainstem in an unbranched fashion at E15, begin emitting collaterals into the brainstem trigeminal nuclei by E17, and form terminal arbors that replicate the patterned array of whisker follicles on the snout (Erzurumlu and Jhaveri, 1992). In wholemount cultures derived from E15 rats, most tissue-specific characteristics of the trigeminal pathway are retained. However, central trigeminal axons do not follow the in vivo differentiation rate, rather, they remain restricted to the central trigeminal, without any collateral branching or arborization during a 3–5-day culture period

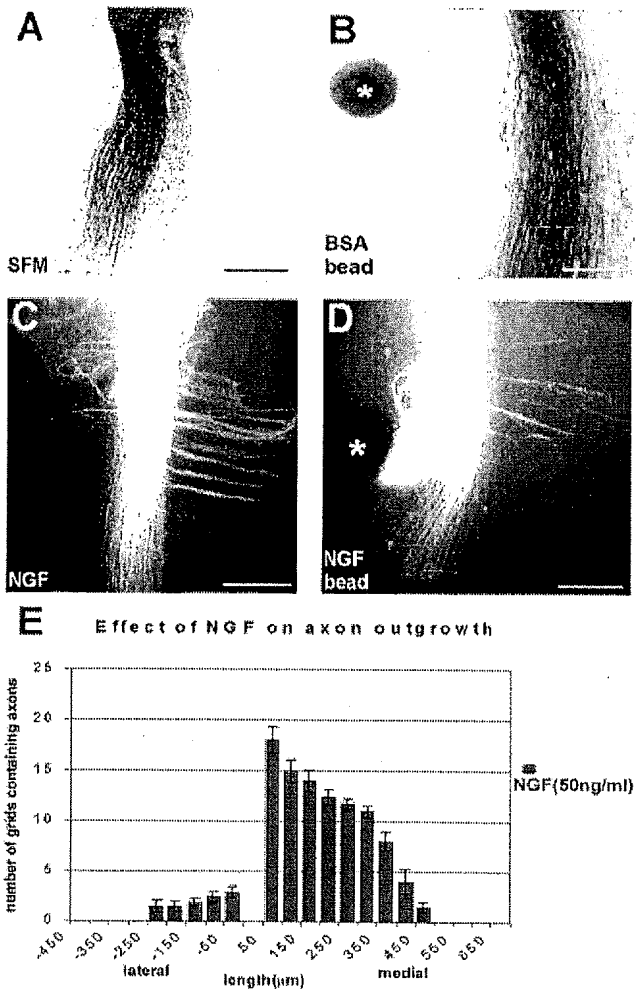

Fig. 2. Axon growth in the central trigeminal tract in wholemount cultures and the effects of neurotrophin treatments. **A:** Unbranched, restricted growth of central trigeminal axons along a narrow pathway within the lateral brainstem in wholemount cultures maintained in SFM. **B:** Similar axon growth in the central trigeminal tract on the side of the brainstem with a control, BSA-soaked bead implant (asterisk). **C:** Effects of exogenous application of NGF in the culture medium on central trigeminal axons. Note the profuse outgrowth outside the tract. **D:** Axon outgrowth outside the central trigeminal tract following NGF-soaked bead implantation along the lateral side of the tract. Note axon growth both toward and away from the source of the neurotrophin. Photomicrographs in A and B are from DiI-labeled cases that have been photoconverted and photographed under lightfield illumination; those in C and D were photographed under epifluorescence by using a rhodamine filter set. In this figure and all the ensuing figures, the control side has been flipped over vertically for better comparison, Thus, in all the figures, medial is toward the right and rostral is up. **E:** Quantitative assessment of axon growth away from the trigeminal tract following NGF treatment. Bar graphs illustrate the number of grids containing axons versus the distance from the central trigeminal tract in lateral (negative) and medial (positive) directions. Error bars represent one standard deviation. Asterisks mark the beads. Scale bars = 200 μm.

(Ulupinar et al., 2000a; see also Fig. 2A). However, when NGF is added to the SFM, many axons grow outside the tract and elongate medially or laterally within the brainstem (n = 8; Fig. 2C; see also Ulupinar et al., 2000a). Quantitative and statistical analyses also indicated that there is more growth medially than laterally within the brainstem explants (Fig. 2E). Central trigeminal tract axons also changed their growth patterns when NGF was presented locally (n = 10; Fig. 2D). A group of axons, close to the bead, left the tract and extended toward it, forming tangles around the NGF source. In addition, some axons grew away from the bead within the brainstem (Fig. 2D). Control beads loaded with BSA had no effect on central trigeminal axons (n = 10; Fig. 2B). To verify that the effect is due to NGF, we added a Trk receptor blocker, K252a (1 μM; Biomol, Plymouth Meeting, PA), to the culture medium containing NGF. In such cases, the effect of NGF was blocked and the central trigeminal tract axons followed a restricted path as if they were cultured in the presence of SFM (data not shown).

Viral infection and axonal morphology

In wholemount trigeminal pathway explants, infection with the GFP-expressing viral constructs only (n = 10; controls) did not affect the morphological features of the central trigeminal axons (data not shown). In such cases, axon growth within the trigeminal tract was indistinguishable from uninfected cases grown in SFM (Fig. 2A). In the presence of NGF in the medium, and following control viral infection with the GFP expression vector alone, central trigeminal axons left the tract and grew unbranched for long distances, as was seen in uninfected cases maintained in the presence of NGF (data not shown). Similar results were also derived from experiments with neurotrophin-soaked sepharose beads that were placed along the lateral side of the trigeminal tract (n = 10). These results indicate that the expression of viral constructs alone does not alter central trigeminal axon growth parameters or their response to neurotrophin treatment. Thus, in the next series of experiments, we used GFP-expressing virus infection on one side of the same wholemount explant culture as a control and GFP-tagged as well as genetically modified Rho GTPase viral constructs on the other side. Central trigeminal axon growth was not altered in cases where TG explants were infected with viral constructs or when LPA was added to the SFM and cultured without any neurotrophins. However, the same constructs or LPA treatment dramatically altered central trigeminal axon growth patterns when neurotrophins were added to the medium or presented locally in sepharose beads.

Rac activity enhances neurotrophin-induced axonal response and is necessary for it

RacAC significantly increased axonal growth outside the trigeminal tract following NGF addition to the culture medium (n = 5; Fig. 3A). Similar effects were also seen when a single NGF-loaded bead was placed along the lateral side of the descending trigeminal tract (n = 12; Fig. 3B). In these cases, numerous axons left the tract at a 90° angle with respect to the rostrocaudal extent of the descending trigeminal tract. Many axons extended toward the bead and engulfed it. Others grew unbranched in the opposite direction away from the bead (Fig. 3B). Comparison of axon growth outside the boundaries of the tract in NGF-stimulated RacAC cases with NGF stimulation alone cases showed a statistically significant increase ($P < 0.01$, df = 11) in axon growth in the former cases where longer distances are covered by axons (Fig. 3C). Axons growing away from the tract were longer (500μm) compared with either noninfected or GFP-infected counterparts (350μm; Fig. 3C). Conversely, viral infections that led to expression of RacDN completely blocked the axonal responses to NGF (n = 12). There were no axons seen leaving the boundaries of the tract medially or laterally following addition of NGF to the medium (Fig. 4A) or placement of NGF beads along the tract (n = 10; Fig. 4B). These cases appeared similar to SFM alone, BSA-loaded bead cases, or controls with only GFP-tagged viral construct infections.

Rho activity blocks NGF-induced axon outgrowth from the trigeminal tract

Addition of LPA (10 μM) to the culture medium containing NGF (50 ng/ml) blocked axonal responses to NGF (n = 10; Fig. 5A) in a similar fashion to that seen with RacDN cases. When NGF-loaded beads were placed along the tract in LPA-treated cases (n = 8), there was some axonal growth toward the bead (Fig. 5B), but not as much as that seen in cases without LPA treatment. In addition, axonal outgrowth away from the source of NGF was highly reduced or eliminated along the central trigeminal tract (Fig. 5B). In some cultures we added a 100 ng/ml dose of NGF (n = 5). Without LPA in the medium, trigeminal tract axons extended beyond the boundaries of the tract and formed a dense, reticular meshwork (Fig. 5C). When LPA was included, this response was largely blocked, and only a few axons were seen to leave the tract in medial or lateral directions (n = 5; Fig. 5D). Statistical analysis of axon growth in these cases also confirmed our observations (Fig. 5E): the axon outgrowth in LPA-treated cases showed a highly significant reduction in axon outgrowth ($P < 0.001$, df = 8).

When trigeminal ganglion neurons were infected with the RhoDN viral construct (n = 10), we observed axon outgrowth away from the tract following NGF stimulation (Fig. 6A). In these cases, there was dense but short (50–100 μm) axon outgrowth medially. Similarly, with NGF-loaded beads (n = 11), there was selective axon growth toward the midline (Fig. 6B). Quantitative comparison of axon outgrowth in NGF (100 ng/ml) only and NGF (100 ng/ml) + RhoDN cases are presented in Figure 6C. F-test analysis verified the equality of variance, and t-test statistics indicated a highly significant decrease in axon outgrowth and an increase in axon number in the first 50 μm columnar distribution ($P < 0.001$, df = 16).

Rac and Rho activity and central trigeminal axonal responses to NT-3 and BDNF

Previous studies in explant and dissociated cell cultures documented that, unlike NGF, NT-3 induces short, but highly branched axonal outgrowth (Lentz et al., 1999; Ulupinar et al., 2000a). In the last series of experiments, we implanted NT-3- or BDNF-soaked beads along the lateral trigeminal tract and examined axon growth patterns following the series of Rac and Rho regulation procedures (n = 10 for each condition) as described above for NGF cases. NT-3 induced short but highly branched axon growth (Fig. 7A). This effect was more pronounced when TG cells were infected with a RacAC viral construct (Fig.

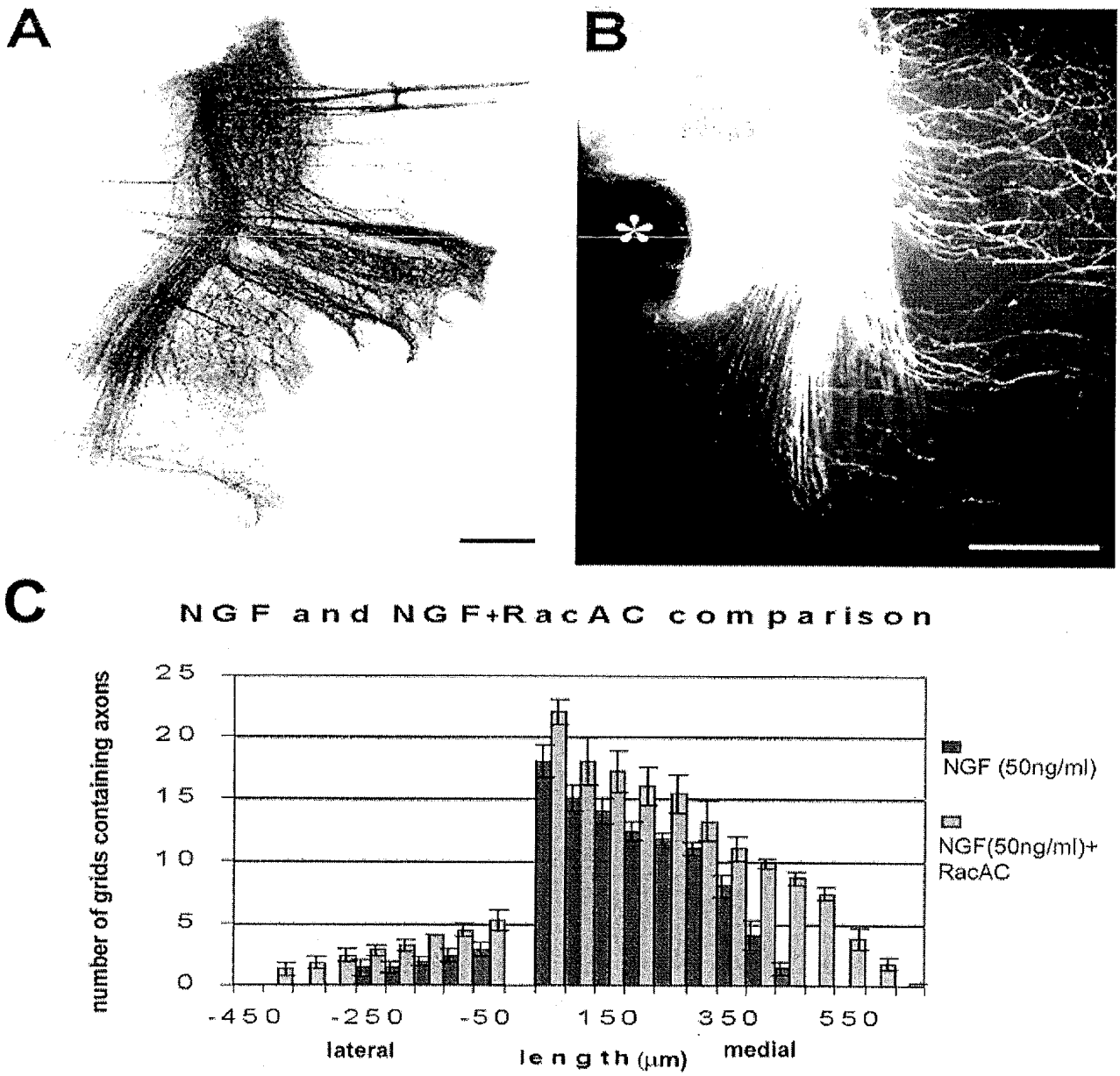

Fig. 3. Axonal responses to NGF following RacAC (RacV12) viral infections. Infection with RacAC significantly increased axonal responses following NGF addition to the culture medium (**A**) or local application of an NGF-soaked bead (**B**). **C:** Quantitative and statistical comparison of axon growth in NGF-treated cases (in the medium) and RacAC-infected cases in the presence of NGF. Bar graphs illustrate the number of grids containing axons versus the distance from the central trigeminal tract in lateral (negative) and medial (positive) directions. Error bars represent one standard deviation. RacAC infection significantly increases axon outgrowth in both lateral and medial directions ($P < 0.01$). Asterisk marks the bead. Scale bar = 200 μm.

7B) and was completely blocked upon Rac DN expressing viral vector infection (Fig. 7C). Suprisingly, LPA treatments led to branched axonal growth both toward and away from the NT-3-loaded bead (Fig. 7D). Rho DN, on the other hand, resulted in branched axonal outgrowth outside the tract away from the source of the neurotrophin much like that seen with NGF cases (Fig. 7E). Similar observations were made following NT-3 addition to the medium without bead implants (data not shown).

BDNF-loaded beads induced axonal growth only toward the bead (Fig. 8A), which was not as branched as that seen in NT-3 cases. Some axons extended long distances, as in

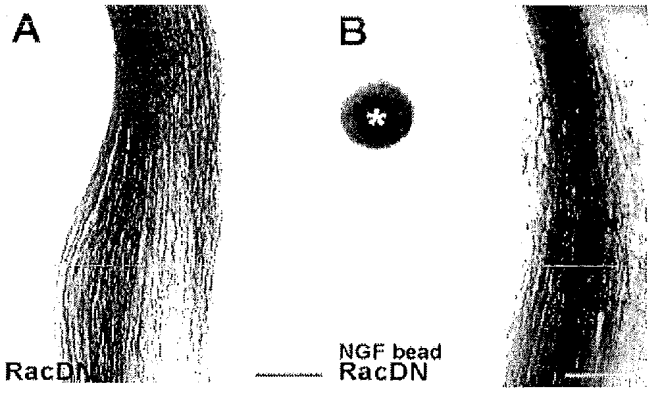

Fig. 4. Axonal responses to NGF following RacDN (RacN17) viral infections. Photoconverted, DiI-labeled central trigeminal axons following RacDN viral infection and NGF stimulation by exogenous (**A**) or local (**B**) supply of the neurotrophin. Note that there are no axons leaving the central trigeminal tract when the ganglion is infected with the RacDN construct. Asterisk marks the bead. Scale bars = 200 μm.

NGF cases. Upon RacAC viral infection, axonal growth toward the bead was increased (Fig. 8B), whereas RacDN eliminated outgrowth completely (Fig. 8C), as seen in NGF and NT-3 cases. Perturbations in Rho activity did not change the direction of axonal outgrowth in BDNF (Fig. 8D), rather, it seemed to change the level of branching at the site of the bead. Upon RhoDN viral infection (Fig. 8D) or Rho activation via LPA (Fig. 8D), there was more branching in the vicinity of the bead.

DISCUSSION

Wholemount explant cultures of the embryonic rodent trigeminal pathway provide an excellent in vitro model system to study mechanisms underlying axon-target interactions. Previous work from our laboratory showed that NGF and NT-3 induce differential growth responses from central trigeminal tract axons (Ulupinar et al., 2000a). Here, we show that the Rho family of GTPases plays a major role in mediating these responses. Neurotrophin-mediated axonal responses of sensory neurons have been emerging from recent in vitro studies and morphological observations from mice with null mutations for specific neurotrophins or Trk receptors. However, in such studies, dissociating between the survival-promoting and axonal differentiation effects of neurotrophins has been a challenge.

Mutant mice that lack the BAX protein have provided some clues within this context. Sensory neurons of these mice survive independently of neurotrophins, and naturally occurring cell death is eliminated to a considerable extent (Deckwerth et al., 1996). In dissociated DRG cultures from BAX null mice, NGF promotes neurite elongation, whereas NT-3 induces, short, highly branched neurites (Lentz et al., 1999). Similar results were obtained for the normal rat trigeminal pathway by using both dissociated trigeminal cell cultures and wholemount explant cultures (Ulupinar et al., 2000a). The specific receptor-ligand interactions and associated intracellular signaling path-

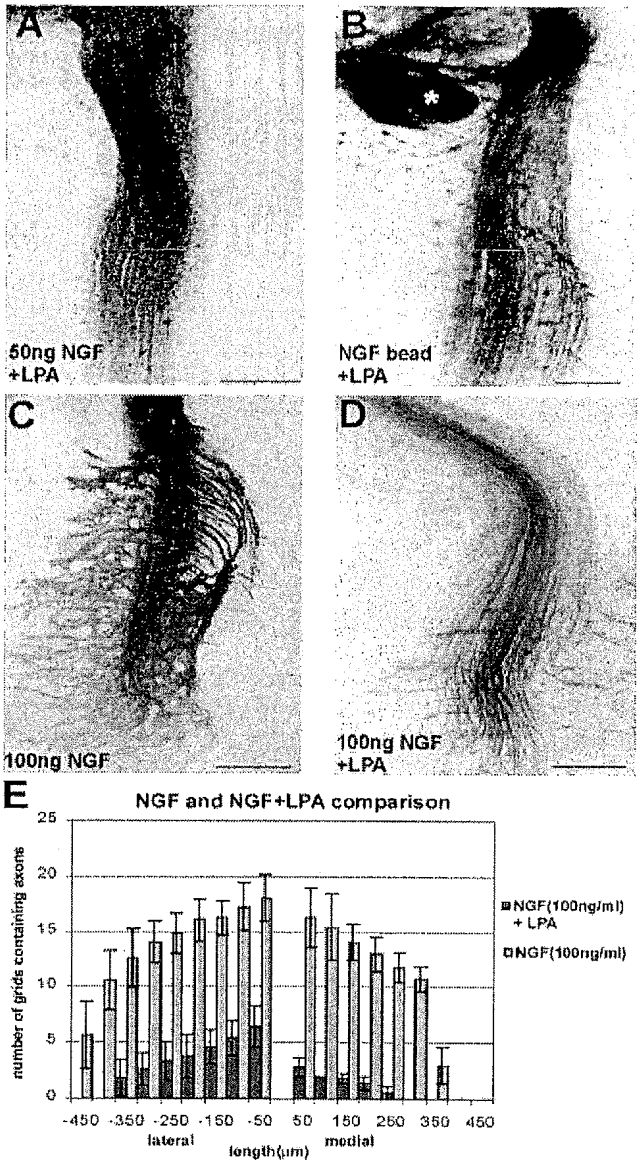

Fig. 5. Effects of LPA treatment on NGF-induced axon outgrowth. LPA added to the culture medium, containing 50 ng/ml NGF, completely blocks axon outgrowth from the central trigeminal tract (**A**). However, relatively low levels of axon outgrowth toward NGF-soaked beads could be seen (**B**). Increased NGF concentration in the culture medium (100 ng/ml) leads to dense axon outgrowth from the tract and formation of axonal tangles (**C**). This effect was largely blocked by LPA (**D**). **E:** Quantitative and statistical comparison of axon growth between cases stimulated with 100 ng/ml NGF and LPA treatment in the presence of NGF (NGF[100 ng/ml] + LPA). Bar graphs illustrating the number of grids containing axons versus the distance from the central trigeminal tract in lateral (negative) and medial (positive) directions. Error bars represent one standard deviation. LPA treatment leads to highly significant reduction in axon outgrowth in both directions ($P < 0.001$). All photomicrographs are from DiI-labeled, photoconverted cases. Asterisk marks the bead. Scale bars = 200 μm.

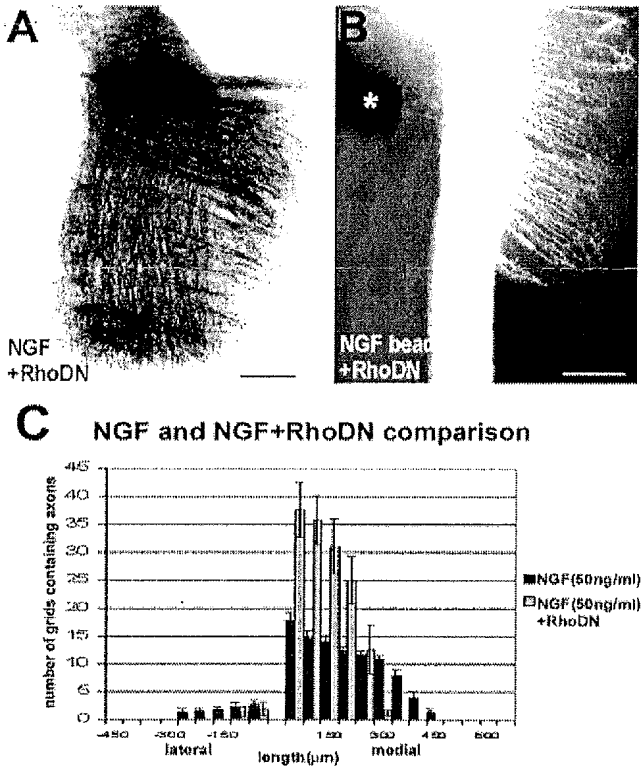

Fig. 6. Reduction of NGF-induced axon outgrowth following RhoDN (RhoN19) viral infections. (**A**; photoconverted) and (**B**) illustrate two exemplary cases one with NGF in the medium and the other with an NGF-loaded bead. Note dense but short axon outgrowth medially away from the central trigeminal tract in both cases. **C**: Quantitative and statistical comparison of axon outgrowth in NGF alone and RhoDN infected cases in the presence of NGF in the medium (NGF + RhoDN). Bar graphs illustrating the number of grids containing axons versus the distance from the central trigeminal tract in lateral (negative) and medial (positive) directions. Error bars represent one standard deviation. RhoDN infection leads to highly significant reduction in axon outgrowth and a higly significant increase in the mean axon number ($P < 0.001$). Asterisk marks the bead. Scale bars = 200 μm.

ways underlying such axonal responses to neurotrophins are presently unclear.

It is also important to note that observations from in vitro experiments do not always reflect actual events in vivo. For example, we recently noted that dissociated E15 rat TG cells all express multiple Trk receptors shortly after they settle down in culture regardless of the presence of neurotrophins in the culture medium (Genc and Erzurumlu, 2000). In contrast, in in vivo or explant cultures, different classes of E15 TG cells express only one high-affinity neurotrophin receptor (Genc and Erzurumlu, 2000; see also Huang et al., 1999a). Although explant cultures reflect in vivo conditions more than dissociated cell cultures, they do not perfectly mimic in vivo progression of developmental events. In the rat, central trigeminal axons begin developing collateral branches and terminal arbors within the brainstem by E17, whereas in explant cultures, E15 central trigeminal axons remain in the elongation phase for 3–5 days after the cultures are set up. The most likely explanation for this is that under culture conditions, maturation of the peripheral or central targets of the TG cells and their axonal projections is delayed. However, TG axons readily respond to neurotrophins and show dramatic changes in their growth characteristics.

Developmental regulation of neurotrophin expression in the peripheral targets of TG axons have been documented in a number of previous studies (Ernfors et al., 1992; Arumae et al., 1993; Buchman and Davies, 1993; Davis et al., 1997). Differential expression of neurotrophins has been noted in the developing spinal cord (Maisonpierre et al., 1990; Elkabes et al., 1994) but has not been carefully examined for the developing brainstem trigeminal nuclei. Thus, the question of whether neurotrophin expression in brainstem trigeminal targets plays a role in shifting central trigeminal axons from elongation to arborization still remains open. It is also likely that temporal regulation of neurotrophin expression in peripheral trigeminal targets could switch central trigeminal axons between different growth patterns. In a preliminary study, such effects were observed within the central trigeminal tract following localized applications of neurotrophin-loaded sepharose beads in the whiskerpad in trigeminal pathway wholemount cultures (Ulupinar et al., 2000b). To date, central trigeminal projections in a variety of presently available neurotrophin or Trk receptor knockout mice have not been examined in detail. In BAX and TrkA double knockout mice, peripheral sensory projections are perturbed, but no abnormalities were noted in the central projections of DRG cells at a gross level (Patel et al., 2000). Future detailed examinations of central trigeminal axon morphologies in BAX plus neurotrophin or Trk receptor double knockout mice could shed light on this issue.

Rac and Rho regulate neurotrophin-induced trigeminal axon outgrowth

The signaling cascade mechanisms behind neurotrophin stimulation have been widely studied (for reviews, see Kaplan and Miller, 1997; Kaplan, 1998; Friedman and Green, 1999; Kaplan and Miller, 2000). Neurotrophins initiate two different signaling cascades for survival and axonal differentiation (for reviews, see Klesse and Parada, 1999; Klesse et al., 1999). There is compelling evidence that Rac activity is involved in the dynamics of axonal and dendritic differentiation (Luo et al., 1996; Threadgill et al., 1997; Ruchuoeft et al., 1999; Li et al., 2000; Nakayama et al., 2000, Tashiro et al., 2000). Here we demonstrate that Rac activity is also required for axonal responses of TG cells to neurotrophic factors.

Rho GTPases regulate cytoskeletal dynamics of growth cones and neurite extension. In a previous study, in vivo introduction of the same viral constructs we used led to dramatic effects on dendritic processes of tectal cells in *Xenopus* (Li et al., 2000) without any application of neurotrophins. However, these viral constructs or LPA treatment did not alter the behavior of central trigeminal axons in the absence of neurotrophins. It is most likely that in the in vitro model of the trigeminal pathway employed in our study, TG cells remain in a quiescent state in comparison with their in vivo counterparts. The fact that they do not collateralize into the brainstem trigeminal nuclei and form terminal arbors in a comparable time period to their in vivo counterparts underscores this. How-

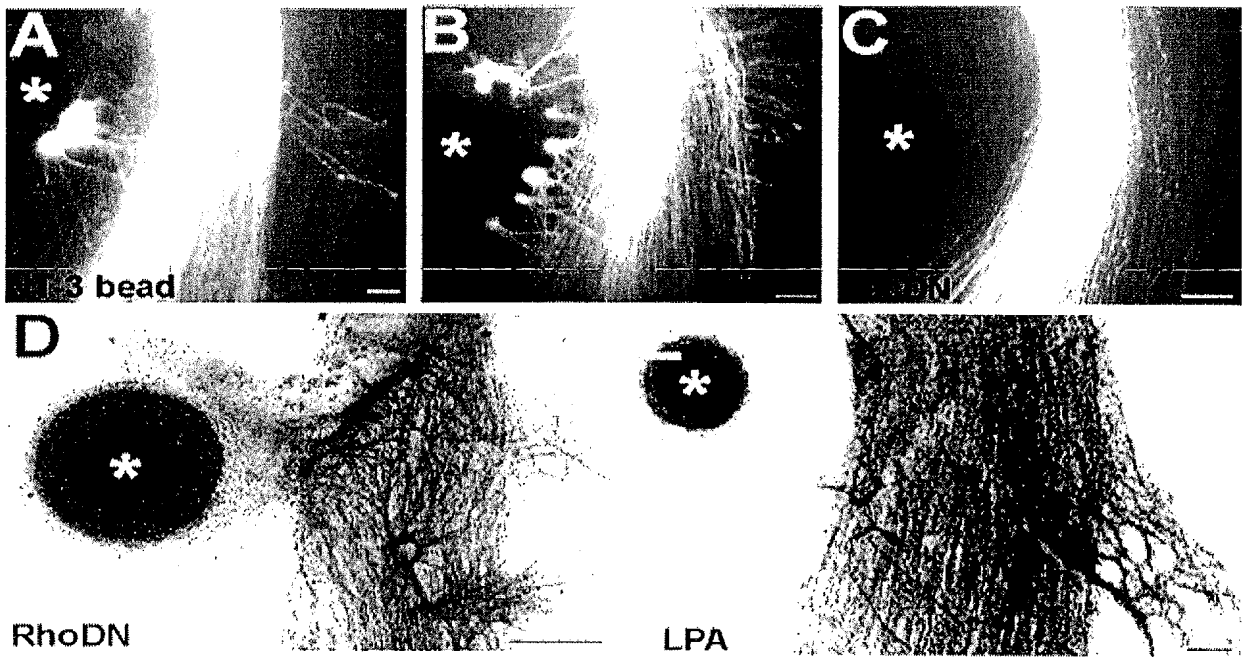

Fig. 7. Axonal responses to NT-3 following Rac and Rho modulation. **A:** Trigeminal axon growth and branching in response to NT-3 soaked beads. **B:** Enhanced response following RacAC. **C:** Such responses are blocked following RacDN viral infections. **D:** RhoDN viral infection decreases axon growth toward the bead. **E:** Activation of Rho induces trigeminal axons to grow medially away from the source of the NT-3 bead; only a few axons grow toward the bead. Asterisks mark the beads. Scale bars = 100 μm.

ever, they readily respond to exogenous or local application of neurotrophins. Our results demonstrate that the Rho family of GTPases regulates this behavior. In the absence of neurotrophic factors, activation or inactivation of one particular Rho GTPase may not be sufficient to change axonal outgrowth properties of the system maintained in vitro.

Rho can bind to the cytoplasmic domain of the p75 receptor in its GTP bound form (Yamashita et al., 1999). Following neurotrophin stimulation, Rho is released to the cytoplasm, where it binds to Rho-GDI and becomes inactive. Inactivation of Rho gives rise to enhanced response to neurotrophic factors (Yamashita et al., 1999). Activation of Rac in the absence of neurotrophic factors did not lead to disruption of the trigeminal tract and axon growth away from it. In addition to what we discussed earlier, this may be due to two other reasons. First, although Rac activity is important for axonal elongation, other cofactors that become available or active upon Trk receptor activation may be required. Second, although Rac is active inside the cell, it may not be able to bind to the downstream signaling proteins due to differences in cellular locations. Docking proteins that become active upon Trk activation might be necessary (Lu et al., 1997).

Rho activity and direction of axonal outgrowth

Activation of Rho induces growth cone retraction (Kozma et al., 1997), alterations in dendritic development (Li et al., 2000; Nakayama et al., 2000), and disruption of axonal path finding (Zipkin et al., 1997). It is suggested that Rho is activated via repulsive axon guidance cues, whereas Rac is activated via attractive cues (Mueller, 1999; Awasaki et al., 2000). Inactivation of Rho initiates neurite outgrowth (Jin and Strittmatter, 1997; Lehmann et al., 1999), and perturbations in Rho activity result in directional changes of growing axons (Awasaki et al., 2000). In our experiments, the RhoDN viral construct induced unidirectional axon growth toward the midline. Despite increased density of axon growth in this direction, axon length was considerably shorter compared with NGF cases alone. Our findings support the idea that Rho is involved in steering growth cones, because upon RhoDN viral infections we observed unidirectional axonal growth toward the midline. Unilateral axon outgrowth from the tract was pronounced with localized application of neurotrophic factors. When NGF and NT-3 were introduced lateral to the central trigeminal tract, RhoDN viral infection led to unidirectional axonal growth toward the midline. This type of outgrowth was not observed in BDNF cases. It is also noteworthy that in several experiments with NGF we observed axon growth toward and away from the source of this neurotrophin. Chemotropic effects of neurotrophins have been recently noted for sensory neurons innervating the limbs (Tucker et al., 2001). A more detailed account of chemotropic and chemorepellent effects of NGF in our system is in preparation as a separate publication.

Balance between Rac and Rho

Recently Trio, a unique GEF that has binding domains for both Rac and Rho, has been studied in detail in *Dro-*

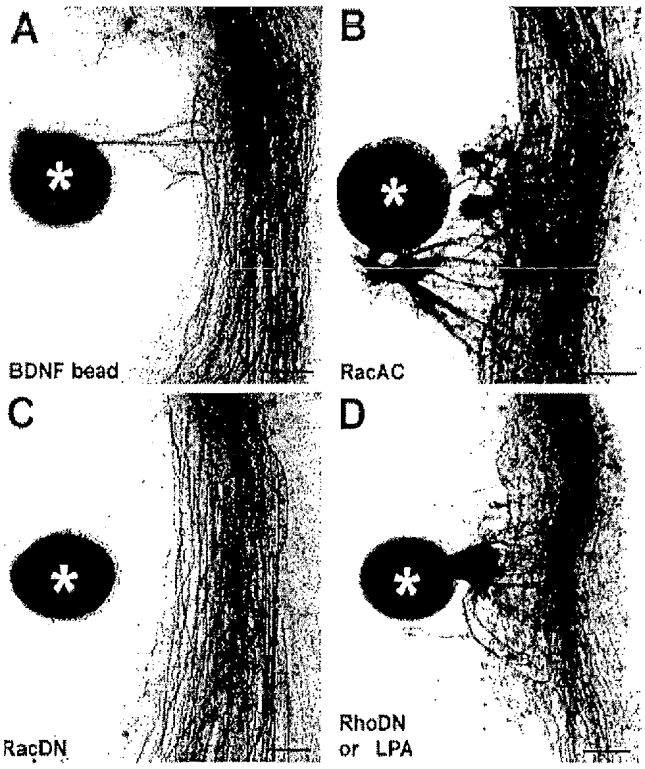

Fig. 8. Axonal responses to BDNF following Rac and Rho modulation. **A:** Trigeminal axon growth toward a BDNF-loaded bead. **B:** Increased axon growth toward BDNF following RacAC. **C:** RacDN completely abolishes response to BDNF. **D:** RhoDN or LPA treatments both have similar effects such that trigeminal axon growth is considerable but lesser than that seen with RacAC cases. Asterisks mark the beads. Scale bars = 100 μm.

sophila (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000). Trio activates Rac selectively by its GEF1 domain, and the GEF2 domain shows specificity for Rho (Debant et al., 1996). Trio is important in mediating axonal development. It has been suggested that Trio is one of the key regulators of Rac and Rho balance inside the cell and that it plays a major role in determining the direction of axonal extension (Lin and Greenberg, 2000). In our culture system, reduction of Rho activity by RhoDN viral infections mimics the situation in which Rho GEFs are reduced in the system. In such cases we observed an abrupt change in direction of axon outgrowth. Activation of Rho, on the other hand, caused decreased axon elongation outside the tract.

Our results underscore the importance of Rac and Rho balance inside the cell for directing axonal outgrowth following neurotrophin stimulation. In in vivo and in whole-mount explant cultures of the trigeminal pathway, the descending trigeminal tract follows a highly restricted route along the lateral brainstem. Development of this pathway, and unbranched elongation of axons within it, most likely results from the interplay of numerous molecular signals in the environment. As in other sensory and motor pathways, some of these signals are positive regulators of axon growth, whereas others are negative regulators, which prevent spillover beyond the boundaries of the tract. Neurotrophin stimulation of axon growth and its regulation via Rac and Rho most likely disrupts the balance of other positive and negative axon guidance signals thereby, disrupting the organization of the central trigeminal tract, as documented in the present study. Intracellular changes in RhoGTPase levels could also alter the responsiveness of trigeminal axons to growth-restrictive cues present in the brainstem outside the trigeminal tract boundaries. Thus, whereas the present results demonstrate the involvement of Rho GTPases in neurotrophin responsiveness of TG axons, they do not rule out altered receptiveness of trigeminal axons to many other signals present in the brainstem.

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